An examination of G_z signalling through multiple phenotypes observed in the $G\alpha_z$ mutant mouse

Kwong Joo Leck

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Developmental Neurobiology Group
John Curtin School of Medical Research
Australian National University

Statement of Authorship

I hereby declare that the contents presented in this thesis are original and all of the experiments were planned and performed by the author under the supervision and guidance of Professor Ian Hendry unless otherwise stated or referenced.

The analysis of sera level of morphine metabolites described in Chapter 3, section 3.2.6, was performed by Andrew Wright and Professor Maree Smith at the University of Queensland. The western blots presented in Figure 3.9 and Figure 5.8 were performed by Professor Ian Hendry and Joan Holgate respectively. The experiment reported in Chapter 5, Section 5.2.4 was performed at Macquarie University in collaboration with Dr. Charles Blaha. Professor David Megirian and Joan Holgate helped with the observations of mice behaviour during the morphine withdrawal and hotplate analgesia experiments described in Chapter 3. Finally, the data in Figure 7.1 were collected in collaboration with Dr. Kim Powell nee Kelleher.

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"If I have been able to see further than others, it is because I've stood on shoulders of giants."

~ Sir Isaac Newton

"Everything in life that is worth getting must be strived, sometimes even fought for."

~ Dr. John Bosco Lee

"Success comes through hardwork, determination, self-discipline, wisdom, the grace of God, the availability of opportunity and a prepared mind to seize the opportunity when it knocks"

~ Maggie Pow

For My Parents

Abstract

This thesis examines the consequences of disrupting the gene that codes for $G\alpha_z$, which results in the absence of signalling through the heterotrimeric G protein G_z in mice. It follows on from my molecular biological work in 1993 on cloning of the mouse $G\alpha_z$ gene, the work of Professor Ian Hendry, Dr. Klaus Matthaei and Albert Mellick in generating the mutant mouse and the work of Dr. Kim Powell (nee Kelleher) on characterization of the mouse phenotype. I have confirmed Dr Kim Powell's earlier finding that the absence of $G\alpha_z$ results in the development of hypertolerance to morphine, by showing the existence of a relationship between the quantity of $G\alpha_z$ present, and the amount of tolerance that the mutant mouse subsequently develops. I have further extended the work to show that this gene dose relationship exists not only when morphine analgesia on the hotplate test was measured, but also when the lethality effects of morphine was considered. I have also demonstrated that the phenomenon occurs in the absence of changes in morphine metabolism in the $G\alpha_z$ knockout mouse. The morphine analgesic tolerance that develops was shown to be non-associative and not due to behavioural learning differences. When physical dependence on morphine was examined in mutant mice, a gene dose dependent reduction in naloxone precipitated jumping was observed without significant differences in the other withdrawal signs. This suggests a dissociation of not only between morphine tolerance and physical dependence in the $G\alpha_z$ knockout mouse, but also between the different symptoms of physical dependence. I have also shown that the absence of $G\alpha_z$ results in a complex alteration of morphine stimulated locomotor responses, suggesting the possible alteration in function of more than one receptor in this intricate behavioural response. I have also examined the locomotor responses of the $G\alpha_z$ knockout mouse to amphetamine, where the mice show an increase in locomotor activity. This, in combination with the published observation by Yang et al. (2000), of an augmented locomotor response of these mice to cocaine, led me to investigate the function of dopamine D2-like receptors in these mice in greater detail. I demonstrated for the first time that dopamine D2-like receptors in the brain can couple to G_z, and a subtle impairment of function of one of the dopamine D2-like receptors (possibly the dopamine D2-short or D4 receptor) may potentially explain the altered locomotor response profiles of the $G\alpha_z$ knockout mouse to psychostimulants and morphine as described above. I have also found that morphine induced hypothermia was

significantly attenuated in the $G\alpha_z$ knockout mouse, and there were strain and species differences in the mechanism of morphine hypothermia. Finally, I have provided a comprehensive review of the literature on what is currently known about the biochemical properties and functions of $G\alpha_z$ and drawn on all my data to suggest some insights into the organization of cellular signalling in an intact cell. These insights lay the theoretical foundation for future discoveries about the functions of $G\alpha_z$ and other signalling molecules *in vivo*.

List of Publications related to this Thesis

Papers published

Kelleher, K.L., Matthaei, K.I., Leck, K.J. and Hendry, I.A. (1998). Developmental expression of messenger RNA levels of the α subunit of the GTP-binding protein, G_z, in the mouse nervous system *Brain Res.* 107: 247-253

Hendry, I.A., Kelleher, K.L., Bartlett, S.E., Leck, K.J., Reynolds, A.J., Heydon, K., Mellick, A., Megirian, D., and Matthaei, K.I. (2000). Hypertolerance to morphine in G(z alpha)-deficient mice. *Brain Res.* 870:10-19

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Abstracts presented at scientific meetings

Hendry, I.A., Heydon, K., Joasoo, S., Leck, K.J., Crouch, M.F., and Matthaei, K. Signal molecules in axons - $G_{z\alpha}$ accumulation after sciatic nerve ligation in mice. *Proc. Aust. Neurosci. Soc.* 5 43 (1994)

Mellick A. S., Leck K. J., Hendry I. A. and Matthaei K. I. Towards a Gzα knockout mouse. *Proc. Aust. Neurosci. Soc.* 7 50 (1996)

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Leck K.J., Bartlett1, S.E., Smith, M.T., Megirian, D., Holgate, J., Matthaei., K.I. and Hendry, I.A.(2001) Development of tolerance to supraspinal morphine analgesia

is dependent on dosage of the heterotrimeric G protein alpha subunit, Gz-alpha. *Proc. Soc. Neurosci.* 224.17

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Leck K.J., Matthaei., K.I. and Hendry, I.A (2002) Alterations in Morphine induced locomotor activation in the Gz-alpha knockout mouse. *Proc.Aust.Neurosci.Soc* 13: 45

List of Abreviations

5HT	serotonin
8OH-DPAT	8-hydroxy-dipropylamino-tetralin
ACTH	adrenocorticotropic hormone
AMP	adenosine monophosphate
ANOVA	Analysis of Variance
ATP	adenosine triphosphate
cAMP	cyclic adenosine monophosphate
CRH	corticotropin releasing hormone
DAG	diacylglycerol
DAMGO	[D-Ala^2,N-MePhe^4,Gly-ol^5]-enkephalin
DPDPE	[D-Pen^2,D-Pen^5]-enkephalin
ED50	median effective dose
ERK	extracellular signal regulated kinase
ES cells	Embryonic stem cells
Eya2	Eyes Absent transcription cofactor 2
G protein	GTP binding protein
GAP	GTPase activating protein
GAP-43	neuronal growth associated protein
GDI	guanine-nucleotide dissociation inhibitor
GDP	guanosine diphosphate
GEF	guanine-nucleotide exchange factor
GPCR	G protein coupled receptor
GRIN	G protein Regulated Inducer of Neurite outgrowth
GTP	guanosine triphosphate
HPLC-MS/MS	high-performance-liquid-chromatography with mass spectrometric detection
IANK	Interleukin-2 activated natural killer
IP_3	inositol-1,4,5-triphosphate
LD50	median lethal dose
M3G	morphine-3-glucuronide
MFB	medial forebrain bundle
NGF	nerve growth factor
Nor-BNI	Nor-binaltorphimine
nor-M3G	normorphine-3-glucuronide
PBS	phosphate buffered saline
PBST	phosphate buffered saline containing 0.1% (v/v) Tween20
PCR	polymerase chain reaction
PLC-β	phospholipase C–beta
PAK1	p21-activated kinase
PVN	paraventricular nucleus
RGS	Regulator of G protein Signalling
SDS	Sodium dodecyl sulphate
U-50,488	hydrochloride (trans-(dl)-3,4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)cyclohexyl]-benzeneacetamide)

Note on change in nomenclature:

I have changed the nomenclature for naming the alpha subunit of the heterotrimeric G protein G_z, from $G_z\alpha$ in earlier publications from my laboratory to $G\alpha_z$ in this thesis. This is to avoid the circularity in the definition of the alpha subunit of G_z since G_z is named based on its alpha subunit.

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Chapter 1

Introduction

1.1 Introduction to heterotrimeric G proteins

1.1.1 G proteins as signal transducers

All living systems need to communicate with their environments, and make appropriate responses to environmental changes in order to survive. The surface of a living cell is constantly bombarded by a myriad of chemical and physical signals. Trans-membrane signalling, the process by which information arriving at the cell surface becomes transmitted to the cell interior, is vital to the proper functioning of all cells. However, the mechanism by which this occurs was not well understood until the pioneering discoveries performed in the laboratories of Martin Rodbell and Alfred Gilman, for which they were jointly awarded the Nobel Prize in Physiology or Medicine in 1994 (http://www.nobel.se/ medicine/laureates/1994/press.html). From their work, we now know that many of the signals impacting on a cell (including light, odorants, and many hormones, chemokines, neurotransmitters and drugs) act on cell surface receptors to cause stimulation of a unique family of membrane-associated proteins called G proteins. G proteins serve as signal transducers. Their role is to relay information arriving at the cell surface to molecular effectors (ion channels, enzymes) within the cell, which will ultimately allow the cell to respond appropriately to changes in the external milieu.

The importance of G proteins in trans-membrane signalling is highlighted by the disastrous consequences that often happen when normal signalling is disrupted. Two well-known examples are whooping cough and diarrhea. Whooping cough occurs when a toxin from the bacterium, *Bordetella pertussis* obstructs the transduction of certain G protein signals, leading to aberrantly high levels of plasma insulin and an acute sensitivity to histamine (Birnbaumer, 1990). Diarrhea is caused by constitutive activation of a G protein signalling cascade in intestinal cells by cholera toxin (produced from the bacterium *Vibrio cholerae*). This results in massive amounts of water and salt to become constantly secreted from affected cells into the lumen of the gut (Spangler, 1992). Besides these toxin-induced diseases, alterations in G protein pathways as a consequence of G protein mutations have produced many other serious illnesses. These

include tumors of the pituitary, adrenal, ovary and thyroid glands (Lyons et al., 1990), cardiac failure, dilative cardiomyopathy (Zolk et al., 2000), ulcerative colitis (Hornquist et al., 1997), night blindness, essential hypertension, McCune-Albright syndrome, pseudohypoparathyroidism (see Farfel et al (1999) and Lania et al (2001) for a review) and insulin resistance, which is characteristic of non-insulin dependent diabetes mellitus (Chen et al., 1997; Moxham and Malbon, 1996). Additionally, alterations in G protein levels have also been observed in bipolar affective disorder (Young et al., 1991; Young et al., 1993) and in drug addiction (McLeman et al., 2000). Given the involvement of G proteins in the etiology of many clinical and psychiatric conditions, it is not surprising that understanding G protein functions is an important goal of modern medical research.

1.1.2 Members of the guanine-nucleotide binding protein superfamily

G proteins derive their names from their ability to bind the guanine nucleotides: guanosine diphosphate (GDP) and guanosine triphosphate (GTP). In this respect, they belong to an expanding superfamily of intracellular proteins whose activities are also regulated by the binding of guanine nucleotides. These other guanine-nucleotide interacting proteins include small GTPases from the Ras, Rho, Rab, Arf and Ran families, which are involved in gene expression regulation, cytoskeletal re-organization, vesicle trafficking and nucleocytoplasmic transport (Matozaki et al., 2000); members of the tubulin protein family, which regulate microtubule assembly and disassembly (Nogales, 2001), GTPases from the initiation and elongation factor families, which are essential for ribosomal protein synthesis (Kaziro et al., 1991) and the dynamin family of large GTPases, which perform vital roles in endocytosis, vesicle trafficking, viral resistance, maintenance of mitochondrial morphology, and cell plate formation in plants (van der Bliek, 1999).

Despite their wide ranging functions, almost all guanine-nucleotide binding proteins share a similar working mechanism (Kaziro et al., 1991). In the inactive form, the protein is bound by GDP. On interaction with a guanine-nucleotide exchange factor (GEF), GDP is released and is replaced by GTP. Binding of GTP causes the protein to assume an active conformation (Sprang, 1997b), which allows it to carry out its prescribed function. This is followed by hydrolysis of GTP to GDP, which returns the protein to its inactive form (Kaziro et al., 1991). Besides GEFs, there are a number of other classes of proteins that can also modulate the activity of guanine-nucleotide

binding proteins. For instance, guanine-nucleotide dissociation inhibitors (GDIs) act to inhibit the guanine-nucleotide exchange reaction and therefore, prevent protein activation. GTPase activating proteins (GAPs), on the other hand, enhance the rate of GTP hydrolysis to GDP, and therefore, accelerate the return of the protein to its inactive configuration (Geyer and Wittinghofer, 1997).

1.1.3 Structure of G proteins

All G proteins comprise three subunits, which has been designated using the Greek letters, α , β and γ. This unique heterotrimeric structure differentiates G proteins from all other guanine-nucleotide binding proteins. Among the three subunits, the α subunits are the largest (39 to 78 kDa) and contain the binding site for guanine nucleotides. The β (35 to 44 kDa) and γ (7 to 9 kDa) subunits form a stable dimer that cannot be dissociated unless the protein is denatured (Clapham and Neer, 1997; Neer, 1995).

The crystallographic structures of the G protein heterotrimer (Lambright et al., 1996; Wall et al., 1995), the inactive and activated forms of the α subunit (Coleman et al., 1994; Coleman and Sprang, 1998; Mixon et al., 1995; Sondek et al., 1994; Sunahara et al., 1997), the $\beta\gamma$ dimer (Sondek et al., 1996) and the activated α subunit in complex with its effector (Slep et al., 2001; Tesmer et al., 1997) have already been solved. The wealth of data from these structural studies, together with the results obtained from biochemical and mutagenesis experiments, have provided tremendous insights into the mechanism of the guanine nucleotide exchange reaction on the α subunit and the way the α, β and γ subunits interact with one another (Neer and Smith, 1996).

Each α subunit is found to have three inter-connected components: a GTPase domain, an alpha-helical domain and an amino terminal helix (Lambright et al., 1996). The GTPase domain comprises a six stranded β sheet surrounded by α helices. These are organized to form five polypeptide loops (G1 to G5), which together constitute the guanine nucleotide binding pocket of the G protein. The domain is evolutionarily conserved among all guanine nucleotide binding proteins and contains distinct sequence motifs responsible for binding the guanine ring, GTP and Mg^{2+} (Hamm and Gilchrist, 1996; Sprang, 1997a). The alpha helical domain consists of five helices arranged to

produce a semi-cylindrical cup surrounding a long central helix. This domain forms a "lid" over the guanine nucleotide binding pocket, and influences the rate of GDP dissociation from the G protein (Hamm and Gilchrist, 1996). The amino terminal helix projects away from the core of the α subunit, and is involved in interaction of the α subunit with the βγ subunit (Conklin and Bourne, 1993).

The β subunit contains an α helix connected to a β propeller that is made up of seven β sheets. These β sheets of the propeller coincide roughly with seven WD40 repeat sequences (Smith et al., 1999) found in the polypeptide chain (Wall et al., 1998). The γ subunit has no tertiary structure, comprising only two major α helices (Wall et al., 1998). The absence of a tertiary structure means that the γ subunit makes very little contact with itself, and almost all its contacts with the β subunit is over an extended hydrophobic groove along the β propeller, and in their amino terminal α helices. The extensive association between the β and γ subunits helps to provide stability to the βγ dimer (Clapham and Neer, 1997).

The three dimensional structures of the various G protein subunits can be visualized on the protein data bank website (http://www.rcsb.org/ pdb) (Berman et al., 2000). The pdb entries are 1TAD and 1TAG for the α subunit, 1TBG for the βγ dimer and 1GOT for the heterotrimeric G protein complex.

1.1.4 The G protein cycle

As with other members of the guanine-nucleotide binding protein superfamily (Section 1.1.2), G proteins function by switching alternately between an inactive GDP bound form and an active GTP bound state.

In the inactive configuration, the α subunit binds GDP and associates with the βγ subunit to form a heterotrimer. The G protein βγ dimer is a guanine nucleotide dissociation inhibitor (GDI) (Higashijima et al., 1987; Sprang, 1997b), and its association with the α subunit helps to stabilize the GDP bound α subunit (Wall et al., 1998), to minimize spontaneous activation of the G protein. In order to activate the G protein, contact of the G protein with a GEF is required. In trans-membrane signalling,

an activated G protein coupled receptor (GPCR) serves as a GEF. G protein coupled receptors are cell surface receptors with seven hydrophobic trans-membrane helices (Morris and Malbon, 1999). The binding of an agonist (e.g. neurotransmitter, hormone) to the receptor produces a conformational change in the receptor (Bourne, 1997) that allows it to interact effectively with its cognate G protein and serve as its GEF (Gudermann et al., 1996). The mechanism by which the stimulated receptor catalyses the exchange of GDP for GTP on the G protein α subunit is still not understood (Hamm, 2001; Marin et al., 2002). Based on the crystallographic structure of the G protein heterotrimer (Lambright et al., 1996; Wall et al., 1995; Wall et al., 1998) and the proposed contact sites between the receptor and G protein (Hamm, 1998), one hypothesis is that the receptor uses the βγ dimer as a lever to pry open the guanine nucleotide binding pocket of the α subunit, causing GDP to be released (Rondard et al., 2001). This dissociation of GDP from the α subunit is the rate limiting step in G protein activation (Ferguson et al., 1986). After the dissociation of GDP, GTP, which is present at a much higher cellular concentration than GDP (Neer, 1995), attaches to the guanine nucleotide binding pocket of the α subunit. The binding of GTP switches the α subunit to its active conformation (Lambright et al., 1996; Sunahara et al., 1997), and in this conformation, the affinity of the α subunit for the βγ dimer (Wall et al., 1998) and for the receptor is assumed to decrease, leading to sequential dissociation of the α subunit and the βγ dimer from the receptor (Neer, 1995). Following this, the GTP-bound α subunit and βγ dimer interact with their respective downstream effectors, and transduce the signal of receptor activation (Birnbaumer, 1990).

Similar to other members of the guanine-nucleotide binding protein superfamily, the G protein α subunit is a GTPase (Kaziro et al., 1991). The α subunit becomes inactivated when its intrinsic GTPase activity hydrolyses the bound GTP to GDP. This most likely occurs via a nucleophilic substitution 2 (S_N2) mechanism, where the γ phosphate of GTP is transferred to water (Sprang, 1997b). The intrinsic GTPase activity of G protein α subunits can be enhanced by interaction with certain effectors (such as phospholipase C-β, type V adenylate cycalse, p115RhoGEF) and a family of proteins known as regulators of G protein signalling (RGS), which function as GAPs (Ross and Wilkie, 2000; Scholich et al., 1999). Since G protein α subunits show wide variations in their intrinsic GTP hydrolysis rates (Fields and Casey, 1997), GAPs can play an important role in determining the time length of signal onset. After GTP hydrolysis, the GDP

bound α subunit re-associates with the βγ dimer, allowing the G protein cycle to continue.

Although the G protein cycle presented above is widely accepted, there is still controversy whether dissociation of G protein into its component α and βγ subunits actually occurs after its activation *in vivo* (Rebois et al., 1997). The original supporting evidences for the subunit dissociation hypothesis have been questioned as they were obtained in solutions containing detergent and high magnesium concentrations (Rebois et al., 1997). When G protein activation is examined under physiological conditions, both α and βγ subunits co-purify in a 1:1 ratio stoichiometrically with one another and with the effector, suggesting dissociation of the βγ subunit from the α subunit might not have occurred (Marbach et al., 1990). Furthermore, a mutant G protein that cannot undergo subunit dissociation, has been found to transduce receptor activation signals, as well as normal G proteins (Klein et al., 2000). On the other hand, many of the amino acid residues on the β subunit that are in contact with the α subunit have been demonstrated to be essential for effector stimulation by the βγ dimer (Ford et al., 1998). Based on this evidence, it is concluded that upon G protein activation, subunit dissociation has to occur to expose these residues concealed by the α subunit to βγ effectors (Ford et al., 1998). This would account for the huge body of empirical data showing both the α and βγ subunits are independently capable of modulating the activities of a vast number of downstream effectors (Clapham and Neer, 1997; Offermanns, 2001).

1.1.5 Diversity of G proteins

The human genome has been estimated to contain twenty-seven α, five β and thirteen γ subunits (Venter et al., 2001). Although the βγ dimer has now been recognized to play an important role in effector activation (Carozzi et al., 1993; Clapham and Neer, 1997; Koch et al., 1994; Logothetis et al., 1987; Tang and Gilman, 1991) and in determining signalling specificity (Dippel et al., 1996; Hou et al., 2000; Johansen et al., 2001; Kleuss et al., 1993; Robillard et al., 2000), G proteins have conventionally been named according to the unique structures of their α subunits. Classification of G proteins based on the amino acid sequence homology among the α subunits has

produced four large G protein families. These are the G_s family, the G_i family, the G_q family and the G_{12} family (Table 1.1).

1.1.5.1 The α subunits

The $G\alpha_s$ family consists of $G\alpha_s$ and the odorant receptor G protein, $G\alpha_{olf}$. $G\alpha_s$ has a large number of possible alternative isoforms (Monteith et al., 1995; Novotny and Svoboda, 1998; Pasolli et al., 2000; Ye et al., 1999) and demonstrates complicated tissue specific imprinting (Li et al., 2000). The protein was originally named based on its ability to stimulate the intracellular enzyme, adenylate cyclase, which catalyses the conversion of adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP) (Kahn and Gilman, 1984). To date, ten different isoforms of adenylate cyclase have been identified in mammals, and $G\alpha_s$ has been found to be capable of stimulating nine of them (Adenylate cyclase types I to IX) (Hanoune and Defer, 2001). The only adenylate cyclase it fails to stimulate is a novel cyclase named soluble adenylate cyclase, which incidentally, is localized to both the soluble and particulate fractions of cells (Zippin et al., 2001) and appears to be resistant to regulation by all G proteins (Buck et al., 1999). Similar to $G\alpha_s$, $G\alpha_{olf}$ also exerts a stimulatory effect on adenylate cyclase activity in a mammalian cell line (Jones et al., 1990; Jones and Reed, 1989) and in neural tissue where $G\alpha_{olf}$ is expressed (Corvol et al., 2001). Another distinguishing feature of all members in this family is that they possess an arginine residue in their GTPase domains that represents the target of adenosine diphosphate (ADP) ribosylation by cholera toxin. Modification of the GTPase domain by the toxin leads to a suppression of the α subunit's intrinsic GTPase activity, resulting in constitutive activation of the G protein (Bourne et al., 1989).

The $G\alpha_i$ family is the largest of the G protein families. All family members, with the exception of $G\alpha_z$ (Casey et al., 1990) and an alternatively spliced variant of $G\alpha_{i2}$ (Montmayeur and Borrelli, 1994), contain a carboxyl terminal cysteine residue, which serves as the substrate for ADP-ribosylation by pertussis toxin (Kaziro et al., 1991). Since the carboxyl terminal of the α subunit is involved in G protein-receptor interaction (Conklin and Bourne, 1993), the structural alteration induced by the toxin disrupts coupling of the G protein to the receptor (Maus et al., 1990), resulting in an

impairment of receptor mediated signalling. The prototypical member of the G_i family is $G\alpha_i$, and it was originally named based on its ability to inhibit adenylate cyclase, and therefore opposed the stimulatory effects of $G\alpha_s$ (Bokoch et al., 1984). However, it was subsequently discovered that only certain isoforms of adenylate cyclases are inhibited by $G\alpha_i$. These are the types I (weak to moderate inhibition), III, V, VI, VIII and IX isoforms of adenylate cyclase (Dessauer et al., 1996; Hanoune and Defer, 2001; Taussig et al., 1994). However, it should be emphasized that not all members of the G_i family cause adenylate cyclase inhibition. Besides the three $G\alpha_i$ isoforms, only $G\alpha_z$ and $G\alpha_o$ have been shown to be capable of inhibiting adenylate cyclase. Specifically, $G\alpha_z$ can inhibit the types I, V and VI isoforms of adenylate cyclase (Ho et al., 2000; Kozasa and Gilman, 1995) while $G\alpha_o$ appear to exert an inhibitory effect only on type I adenylate cyclase (Taussig et al., 1994).

The $G\alpha_q$ family consists of five members. All of them share the capacity to stimulate isozymes belonging to the phospholipase C–beta (PLC-β) family (Lee et al., 1992; Offermanns and Simon, 1995; Wu et al., 1992). Activation of PLC-β cleaves the phospholipid, phosphatidylinositol-4,5-biphosphate into the intracellular messengers, inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) (Neves et al., 2002). IP_3, in turn triggers the release of calcium from intracellular stores, and the consequent rise in cytosolic calcium, recruits protein kinase C to the plasma membrane. At the membrane, DAG in combination with calcium, causes the activation of protein kinase C (Alberts et al., 1994; Asaoka et al., 1992), a serine-threonine kinase with diverse functions in the cell (Liu and Anand, 2001; Ventura and Maioli, 2001). Besides activating the protein kinase C cascade, members of the G_q family may also trigger other signalling cascades through the stimulation of protein tyrosine kinase 2 (Della Rocca et al., 1997; Shi and Kehrl, 2001).

The $G\alpha_{12}$ family contains only 2 members, $G\alpha_{12}$ and $G\alpha_{13}$. Although $G\alpha_{12}$ and $G\alpha_{13}$ appear to be expressed ubiquitously (Hepler and Gilman, 1992), the signal transduction pathways mediated by $G\alpha_{12/13}$ *in vivo* remain poorly understood as most experiments investigating $G\alpha_{12/13}$ have been performed using transfected cell lines (Neves et al., 2002). However, mice deficient in $G\alpha_{12}$ and $G\alpha_{13}$ have been produced and analyses of these mice have provided some insights into the functions of these G proteins (Offermanns, 2001). The loss of $G\alpha_{13}$ in mice causes embryonic lethality, probably

resulting from a failure of angiogenesis in the yolk sac and abnormal vascular organization in the head mesenchyme. Additionally, thrombin induced cell migratory responses was absent in $G\alpha_{13}$ deficient fibroblasts (Offermanns et al., 1997). Subsequent studies establish that $G\alpha_{13}$ directly stimulates p115RhoGEF (Hart et al., 1998), the GEF for the small G protein, Rho. Rho plays an important role in the regulation of the actin cytoskeleton and in cell motility (Sah et al., 2000), and it is hypothesized that altered Rho signalling is likely to have contributed to the cell migration defects observed in the $G\alpha_{13}$ knockout animals (Offermanns, 2001). A deficiency of $G\alpha_{12}$ in mice produces no obvious phenotype, although intriguingly, $G\alpha_{12}$ knockout mice that are simultaneously deficient in $G\alpha_q$ or heterozygous for $G\alpha_{13}$ die *in utero* (Gu et al., 2002). This suggests that $G\alpha_{12}$ is involved in mouse embryonic development, although its deficiency can be compensated for by activation of either $G\alpha_q$ or $G\alpha_{13}$.

1.1.5.2 The βγ subunits

In addition to the α subunits, the β and γ subunits are increasingly recognized to contribute to the diversity of G protein functions. For instance, the activities of various G protein gated inwardly retifying potassium channels (Dascal, 1997) and voltage dependent calcium channels (Zamponi and Snutch, 1998) have been found to be principally regulated by the βγ subunits. At present, five β and thirteen γ subunits, each with a distinctive tissue distribution, are known to exist (Table 1.2). Compared to the γ subunits, the β subunits are less heterogeneous. For instance, $G\beta_1$ to $G\beta_4$ share more than 80% amino acid sequence similarity (Gautam et al., 1998) and they have a ubiquitous expression, which suggests many are probably co-expressed in the same cells. This has led to the suggestion that β subunits are likely to have a limited ability to generate diversity or to account for signalling specificity (Vanderbeld and Kelly, 2000). On the other hand, it may be that the different co-expressed β subunits are non-redundant and each of them may preferentially interact with distinct α and γ subunits, as well as receptors and effectors. In congruence with this, differences between the four β subunits in transducing the effects of various hormones on downstream effectors have been observed (Johansen et al., 2001). Moreover, the discovery of $G\beta_5$ and its alternatively spliced variant $G\beta_{5(long)}$, which share only 53% amino acid sequence homology with the other Gβ isoforms (Maier et al., 2000), and which are uniquely

expressed in nervous tissues and in the retina respectively (Watson et al., 1994; Watson et al., 1996; Zhang et al., 2000), will add to the contribution made by the β subunit to G protein diversity. Consistent with this idea, the $G\beta_5$ subunit has been found to discriminate between different receptor and effector subtypes (Maier et al., 2000; Robillard et al., 2000).

The γ subunit is the smallest of the three G protein subunits. Despite its small size, the thirteen currently known γ subunits demonstrate considerable diversity in their amino acid sequence (Vanderbeld and Kelly, 2000), and can be classified based on amino acid homology into four subfamilies (Table 1.2). All γ subunits are post-translationally modified by the attachment of an isoprenoid group to a cysteine residue four amino acids from the carboxyl terminal, followed by proteolytic cleavage of the three terminal residues and finally, methylation of the exposed prenylcysteine (Higgins and Casey, 1996). The isoprenoid group attached is either a fifteen carbon farnesyl group or a twenty carbon geranylgeranyl group depending on the nature of the last amino acid at the carboxyl terminal. If the terminal amino acid is a leucine residue, geranylgeranylation occurs (Moores et al., 1991). However, if it is serine, glycine, alanine, cysteine, threonine, histidine, asparagine or glutamine, farnesylation occurs (Moores et al., 1991). Members of the γ1 subfamily have a serine residue at their carboxyl termini and are therefore farnesylated, while all other known γ subunits have a terminal leucine residue and are geranylgeranylated (Gautam et al., 1998). Prenylation of the γ subunit is essential for association of the βγ dimer with the α subunit (Higgins and Casey, 1994; Matsuda et al., 1994; Matsuda et al., 1998) and the nature of the prenylation (whether farnesylation or geranylgeranylation) can influence the strength of the interaction between the βγ dimer with the plasma membrane, as well as with G protein receptors and effectors (Balcueva et al., 2000; Fogg et al., 2001; Gautam et al., 1998; Matsuda et al., 1998). Accordingly, G proteins that contain γ subunits belonging to the γ1 subfamily may interact with receptors (Butkerait et al., 1995; Kisselev et al., 1995) and effectors (Iniguez-Lluhi et al., 1992) differently from G proteins that contain γ subunits from other γ subfamilies. Besides the carboxyl terminal, other regions of the γ subunit may also be involved in determining the specificity of G protein interaction with receptors and effectors (Lim et al., 2001; Myung et al., 1999). For instance, the γ1 and γ11 subunits are both farnesylated and share very similar carboxyl terminal amino

acid sequences, yet the α_{2a} adrenergic receptor prefers G proteins containing the $\gamma 11$ subunit over those containing the $\gamma 1$ subunit (Richardson and Robishaw, 1999).

Taken together, although G proteins are often referred to base on the identity of their α subunits, it is clear from the above review that all three subunits in the heterotrimer contribute to the interaction of the G protein with receptors and effectors. Accordingly, the functional consequences of G protein activation depend on the combination of α, β and γ subunits making up the G protein that associates with a particular receptor. If each of the twenty-seven currently known α subunits is able to combine with any of the other distinct β and γ subunits to form a heterotrimer, this would result in (27 x 5 x 13) 1755 possible G proteins. However, random associations between the subunits do not occur in nature (Exner et al., 1999; Kontani et al., 1992; Lee et al., 1995; Pronin and Gautam, 1992; Vanderbeld and Kelly, 2000). Due to selectivity between certain α and γ subunits for one another (Rahmatullah et al., 1995) and structural compatibility between the β and γ subunits (Wall et al., 1998), as well as their differential tissue distribution (Tables 1.1 and 1.2), specific combinations of subunits have been observed to interact preferentially with one other (Asano et al., 1999; Exner et al., 1999), resulting in a more limited pool of G proteins. Nonetheless, this 'limited' pool of G proteins is still large, and identifying the specific $\alpha\beta\gamma$ composition of a G protein will be important for defining the function of a particular G protein.

1.1.6 Regulators of G protein Signalling

Besides contacting receptors and effectors, G proteins also interact with members from the Regulator of G protein Signalling (RGS) family. RGS proteins represent a large family of proteins coded in humans by twenty seven different genes (Venter et al., 2001). They share a characteristic RGS domain that is about 130 amino acids in length, which can interact with Gα subunits (Zheng et al., 1999). Based on sequence homology within their RGS domains, the currently known RGS proteins have been grouped into five subfamilies (Table 1.3). With the exception of axin and conductin from the RA subfamily, all RGS proteins are capable of stimulating the intrinsic GTP hydrolysis rate of Gα subunits, and hence, function as GAPs (Ross and Wilkie, 2000). On top of this, there are a number of other molecules containing domains homologous to RGS domains

Table 1.1: The mammalian heterotrimeic G protein families

Family	α subunit	Mass (kDa)	Toxin [a]	Tissue Distribution
G_s	α_s (short) [b]	44.2	CTX	Ubiquitous
	α_s (long) [b]	45.7	CTX	Ubiquitous
	α_s (XL) [c, 4]	78[5]	CTX[5]	Neuroendocrine tissues (pituitary, adrenal medulla, pancreas)[4,6], cerebellar purkinje cells[4]
	α_{olf}	44.7	CTX	Olfactory epithelium, Neuronal[7] (striatum, dentate gyrus, medial habenula), heart[8]
G_i	α_{t1}	40	CTX, PTX	Retinal rods, taste buds[9]
	α_{t2}	40.1	CTX, PTX	Retinal cones, kidney[10], pancreas[11], pituitary[11], adrenal[11]
	α_{gust}	40.5	PTX [d]	Taste buds
	α_{i1}	40.3	PTX	Widely expressed
	α_{i2} [e]	40.5	PTX [e]	Ubiquitous
	α_{i3}	40.5	PTX	Widely expressed
	α_{o1} [f]	40	PTX	Brain and others
	α_{o2} [f]	40.1	PTX	Brain and others
	α_z	40.9		Brain and others[11]
G_q	α_q	42		Ubiquitous
	α_{11}	42		Widely expressed
	α_{14}	41.5		Kidney, lung, spleen, testis, liver, pancreas[11]
	α_{15}	43		Hematopoietic cells
	α_{16}	43.5		Hematopoietic cells[17]
G_{12}	α_{12}	44		Ubiquitous
	α_{13}	44		Ubiquitous

[a] Sensitivity of the G protein α subunits to modification by cholera toxin (CTX) or pertussis toxin (PTX).
[b] Both the long and short isoforms of α_s possess a number of alternately spliced variants[1,2,3].
[c] The extra large isoform of α_s, α_s (XL) consists of at least 3 alternately spliced isoforms[6].
[d] α_{gust} (gustducin) is a substrate for pertussis toxin[12]. Its sensitivity to cholera toxin has not been investigated although the arginine residue (R178) that serves as the site of ribosylation by the toxin is present in the protein[13].
[e] α_{i2} has two alternatively spliced froms[14]. The spliced variant of α_{i2} that is localized to the golgi apparatus is likely to be resistant to pertussis toxin as it lacks the cysteine residue, which is the substrate of adenosine diphosphate (ADP) ribosylation by the toxin[14].
[f] Two alternate forms of α_{o1} and α_{o2} have been found in the brain [15,16].

This table is compiled using the information from Hepler and Gilman (1992) (Pg. 384, table 1) and Offermanns (2001) (pg. 1636, table 1), with the following additional references: (Monteith et al., 1995) [1], (Ye et al., 1999)[2], (Novotny and Svoboda, 1998)[3], (Kehlenbach et al., 1994)[4], (Klemke et al., 2000)[5], (Pasolli et al., 2000)[6], (Herve et al., 2001)[7], (Ferrand et al., 1999)[8], (Ruiz-Avila et al., 1995)[9], (Yamaguchi et al., 1997)[10], (Zigman et al., 1994)[11], (Hoon et al., 1995)[12], (McLaughlin et al., 1992)[13], (Montmayeur and Borrelli, 1994)[14], (McIntire et al., 1998b)[15], (McIntire et al., 1998a)[16], (Amatruda, III et al., 1991)[17].

Table 1.2: Diversity of G protein βγ subunits

Subfamily	Subunit	Mass (kDa)	Tissue Distribution
	β_1	37.3	Ubiquitous
	β_2	37.3	Widely expressed
	β_3 [a]	37.2	Widely expressed
	β_4	37.2	Widely expressed
	β_5 [a]	39 / 44 [10]	Brain and spinal cord (β_5) Retina (β_{5-long})[1]
γ_1	γ_1	8.4	Retinal rods
	γ_{8cone}	7.7	Retinal cones
	γ_{11}	8.5	Heart, lung, spleen, placenta, pancreas, skeletal muscle [2]
	γ_{14}	7.7	Heart, liver, skeletal muscle[2]
γ_2	γ_2	7.9	Brain, adrenal gland, testis, white blood cells, lung[3]
	γ_3	8.5	Brain (neurons[4]) and testis
	γ_4	8.4	Brain, lung, skeletal muscle, kidney, pancreas[5]
	γ_7	7.5[6]	Brain, heart, lung, , kidney, spleen[6]
	γ_{8olf}	7.8	Olfactory and vemeronasal neurons
	γ_{12}	7.9[11]	Ubiquitous (Glial cells in the brain[4])
γ_5	γ_5	7.3	Liver, heart, kidney, lung, brain[6], neuronal precursor cells[7]
	γ_{10}	7.2	Ubiquitous[5]
γ_{13}	γ_{13}	7.9	Thalamus, retina and taste buds[8]

[a] The β_3 subunit has an alternately spliced shorter variant, β_3-short [9],while the β_5 subunit has an alternately spliced longer variant, β_5-long[10].

This table is prepared using the information from Hepler and Gilman (1992) (Pg. 384, table 2) and Vanderbeld and Kelly (2000) (Pg. 539, Table 1) with the following additional references: (Zhang et al., 2000)[1], (Balcueva et al., 2000)[2], (Modarressi et al., 2000)[3], (Morishita et al., 1997)[4], (Ray et al., 1995)[5], (Cali et al., 1992)[6], (Asano et al., 2001)[7], (Blake et al., 2001)[8], (Virchow et al., 1999)[9], (Watson et al., 1996)[10], (Morishita et al., 1995)[11].

Table 1.3: The mammalian RGS protein family

Subfamily	Protein	Tissue Distribution	Protein Interaction domains
RZ	RGSZ1	Brain	RGS
	RET-RGS1	Retina	RGS
	RGSZ2	Not determined	RGS
	GAIP	Ubiquitous, low in brain	RGS, PDZ
R4	RGS1	B-lymphocytes, lung	RGS
	RGS2	Ubiquitous	RGS
	RGS3	Ubiquitous	RGS
	RGS4	Brain, heart	RGS
	RGS5	Ubiquitous	RGS
	RGS8	Brain	RGS
	RGS13	Lung	RGS
	RGS16	Ubiquitous	RGS
R7	RGS6	Brain	RGS, GGL, DEP
	RGS7	Brain, B-cells	RGS, GGL, DEP
	RGS9	Retina, neurons	RGS, GGL, DEP
	RGS11	Brain	RGS, GGL, DEP
R12	RGS10	Brain	RGS
	RGS12	Brain, spleen, lung, testis	RGS, RBD, PDZ, PTB, GPR
	RGS14	Brain, spleen, lung	RGS, RBD, GPR
RA	Axin	Ubiquitous	RGS, APC, GSK, PP2A, Cat, DIX
	Conductin	Lung, thymus	RGS, APC, GSK, PP2A, Cat, DIX

This table is compiled based on information provided in Ross & Wilkie (2000) and De Vries, L. et al. (2000). The abbreviations for protein interaction domains referred to in the table are: RGS (Regulator of G protein Signalling domain), PDZ (PSD95/Dlg/ZO-1 homology domain), GGL (Gγ-like domain), DEP (Dishevelled/EGL-10/pleckstrin homology domain), RBD (Ras/Rap binding domain), GPR (G protein regulatory motif or GoLoco binding motif), PTB (Phosphotyrosine binding domain), APC (Adenomatous Polyposis Coli protein binding motif), GSK (Glycogen Synthase Kinase 3β binding domain) PP2A (Protein Phosphatase 2A binding domain), Cat (β Catenin binding domain) and DIX (Dishevelled and Axin homology domain).

that also allow them to interact with Gα subunits. These include the Rho selective guanine nucleotide exchange factor, p115RhoGEF, which function as GAPs for both $G\alpha_{12}$ and $G\alpha_{13}$ (Kozasa et al., 1998) and G protein coupled receptor kinase 2 (GRK2), which inhibits $G\alpha_q$ mediated phospholipase C -β activity through sequestration of activated $G\alpha_q$ (Kozasa, 2001).

Several larger RGS proteins contain protein interaction domains outside their RGS domains (Table 1.3). These protein interaction domains may help to link G protein signalling to other signalling cascades within the cell (De Vries et al., 2000). For instance, the presence of a Ras/Rap binding motif in RGS12 and RGS14 might allow these two RGS proteins to become the center of two signalling networks that involve G proteins and Ras/Rap GTPases (Ponting, 1999). Additionally, these other protein interaction domains may also play a direct role in regulating G protein function. The G protein regulatory domain (also known as the GoLoco binding domain) is found in RGS12 and RGS14, and the domain has been shown to bind specifically to the α subunits of some $G\alpha_i$ family members to inhibit GDP release (Hollinger et al., 2001; Kimple et al., 2002). Therefore, RGS12 and RGS14 can serve as guanine nucleotide dissociation inhibitors (GDIs) (Hollinger et al., 2001; Kimple et al., 2001) in addition to their role as GAPs, and this would be expected to severely dampen the signalling of those G protein α subunits they interact with. On the other hand, RGS proteins from the R7 subfamily contain a 64 amino acid region, called a G-gamma-like domain. This G-gamma-like domain, originally identified based on its sequence similarity to G protein γ subunits, has been found to bind $G\beta_5$ subunits specifically (Snow et al., 1998). In phospholipid vesicles, RGS9 (a member of the R7 subfamily) is capable of associating with $G\beta_5$ and $G\alpha_o$ to form a heterotrimer and support receptor stimulated guanine nucleotide exchange on the $G\alpha_o$ subunit (Sondek and Siderovski, 2001). In *Xenopus* oocytes, co-expression of $G\beta_5$ with RGS7 (another member from the R7 subfamily) or RGS9 lead to acceleration of the kinetics of coupling between m2 muscarinic acetylcholine receptor and G protein coupled inward rectifying potassium channels (Kovoor et al., 2000). Since $G\beta_5$ and RGS proteins from the R7 subfamily are predominantly expressed in nervous tissues (De Vries et al., 2000; Watson et al., 1994), their association with one another and with Gα subunits could produce a heterotrimer with rapid inactivation kinetics (due to GAP activity on the RGS protein), and contribute to the rapidity and specificity of neuronal signalling.

1.1.7 Covalent modifications of G proteins

G protein subunits are subjected to a number of co-translational and post-translational modifications (Chen and Manning, 2001; Dunphy and Linder, 1998). These modifications play an important role in determining the properties of the G protein, which could sometimes lead to alteration of their functions. Two examples already mentioned are ADP-ribosylation of α subunits from the $G\alpha_s$ family by cholera toxin causing prolonged G protein activation, and ribosylation of α subunits from the $G\alpha_i$ family by pertussis toxin, leading to disruption of receptor-G protein coupling.

On the other hand, most covalent modifications appear to be essential for the G protein to function normally. Prenylation refers to the attachment of one or more isoprenyl groups (either a fifteen carbon farnesyl group or a twenty carbon geranylgeranyl group) to cysteine residues near the carboxyl terminal of a protein (Bhatnagar and Gordon, 1997). The attachment takes place via a stable thioether bond, and occurs in all G protein γ subunits (Gautam et al., 1998). This lipid modification is a pre-requisite for the βγ subunits to associate with the α subunit to form the G protein heterotrimer (Higgins and Casey, 1994), and plays a key role in targeting both the βγ dimer (Simonds et al., 1991) and the α subunit (Fishburn et al., 2000) to the membrane.

The α subunits are also dependent on lipid modifications to assist them in their localization to the membrane. Myristoylation refers to the attachment of a fourteen carbon myristate group to a glycine residue on a protein via an amide bond (Bhatnagar and Gordon, 1997). This reaction happens co-translationally and is catalysed by the enzyme myristoyl CoA: protein N-myristoyltransferase, which specifically recognizes an amino terminal MGXXXS motif unique to α subunits from the $G\alpha_i$ family (Chen and Manning, 2001). Therefore, only $G\alpha_i$ family members are myristoylated and myristoylation results in anchorage of the α subunit to the membrane, so that they can interact with their cognate receptors (Morales et al., 1998; Mumby et al., 1990). For α subunits from other G protein families, lipid modification takes place via palmitoylation. Palmitoylation refers to the post-translational attachment of palmitic acid (a sixteen carbon saturated fatty acid) to cysteine residues near the amino terminals of proteins (Bhatnagar and Gordon, 1997). The majority of G protein α subunits are

palmitoylated, and the process occurs via a reversible thioester bond (Dunphy and Linder, 1998). Palmitoylation increases the hydrophobicity of the α subunits and helps in stabilizing their association with the plasma membrane (Fishburn et al., 2000). This may be particularly important for α subunits that are not myristoylated since attachment of G proteins to the membrane is thought to require at least two hydrophobic signals, one from the isoprenyl group on the γ subunit and the other from the α subunit (Dunphy and Linder, 1998; Fishburn et al., 1999; Morales et al., 1998). Additionally, palmitoylation of the α subunit has been found to increase the affinity of the α subunit for the βγ subunit (Iiri et al., 1996) and block the GAP activity of RGS proteins (Tu et al., 1997). Reversible palmitoylation may therefore play a key role in regulating the interaction of the α subunit with the βγ subunits and with RGS proteins.

Protein phosphorylation and dephosphorylation represent another covalent modification whereby the functions of a G protein can be regulated. Phosphorylation has been observed to occur on the serine residues of $G\alpha_z$, $G\alpha_{12}$ and $G\gamma_{12}$ by protein kinase C, on the tyrosine residues of $G\alpha_i$, $G\alpha_s$ and $G\alpha_q$ by tyrosine kinases and on a histidine residue of Gβ possibly via a nucleoside diphosphate kinase (Chen and Manning, 2001). Protein phosphorylation could alter the strength of the association between α and βγ subunits (Fields and Casey, 1995; Kozasa and Gilman, 1996; Morishita et al., 1995), and influence the interaction of the phosphorylated G protein subunit with downstream effectors (Yasuda et al., 1998) and RGS proteins (Wang et al., 1999).

1.2 $G\alpha_z$: a G protein α subunit with unique biochemical properties

1.2.1 $G\alpha_z$: a member of the $G\alpha_i$ family

$G\alpha_z$ was discovered in 1988 by reduced stringency hybridization of a bovine $G\alpha_{t1}$ and a rat $G\alpha_{i2}$ probe to cDNA libraries from human retina and rat brain respectively (Fong et al., 1988; Matsuoka et al., 1988). Based on sequences that are conserved between the human and rat $G\alpha_z$ cDNAs and at the same time, differ maximally from the sequences of other Gαs, we designed a pair of polymerase chain reaction (PCR) primers, which allowed the amplification and subsequent cloning of the corresponding partial mouse $G\alpha_z$ cDNA from cerebellum (Leck, 1993). The full mouse $G\alpha_z$ cDNA (Genebank

accession number: XM_109580) has recently become available and its sequence matches exactly with that of our partial mouse $G\alpha_z$ cDNA (Genebank accession number: AF059672). Comparison of the translated mouse $G\alpha_z$ protein sequence with that from human and rat shows the three polypeptides share greater than 98% sequence identity, confirming that they are the same protein.

The amino acid sequence of $G\alpha_z$ is most similar to that of the $G\alpha_i$s, leading to the classification of $G\alpha_z$ as a member of the $G\alpha_i$ family (Simon et al., 1991). $G\alpha_z$ shares 66-67% identity with $G\alpha_{i1}$, $G\alpha_{i2}$ and $G\alpha_{i3}$, 59-60% identity with $G\alpha_o$ and 55-57% identity with $G\alpha_t$ (Matsuoka et al., 1988).

1.2.2 Unique features in the $G\alpha_z$ protein sequence

Although $G\alpha_z$ is a member of the $G\alpha_i$ family, the rather low sequence identity between $G\alpha_z$ and other $G\alpha_i$ family members suggest $G\alpha_z$ may possess characteristics that differ from other family members. Analysis of the polypeptide sequence of $G\alpha_z$ reveals a distinctive GTSNS sequence in its GTPase domain that replaces a consensus GAGES motif found in other members of the $G\alpha_i$ family (Figure 1.1, Panel A). When an analogous GTSNS mutation is made in $G\alpha_s$, the mutant exhibits a fifty fold reduction in GTPase activity compared to wildtype $G\alpha_s$ (Casey et al., 1990). One may therefore expect $G\alpha_z$ to show a sluggish rate of GTP hydrolysis, which will make its active GTP-bound conformation relatively stable when the protein becomes activated. Another unique feature in the $G\alpha_z$ polypeptide sequence is the substitution by isoleucine of a cysteine residue located four amino acids from the carboxyl terminal ends of other $G\alpha_{i/o}$ proteins (Figure 1.1, Panel B). This cysteine residue is the target of ADP ribosylation by pertussis toxin, which disrupts receptor-G protein coupling by causing structural changes to the carboxyl terminals of $G\alpha_{i/o}$ subunits (Dratz et al., 1993). The absence of the cysteine residue is expected to make $G\alpha_z$ resistant to modification by the toxin.

1.2.3: $G\alpha_z$ has a sluggish GTPase activity

Predictions about the biochemical properties of $G\alpha_z$ based on information available in

Panel A:

$G\alpha_z$	39-46:	L G **T** **S** **N** S G K S
$G\alpha_{i1}$	39-46:	L G A G E S G K S
$G\alpha_{i2}$	39-46:	L G A G E S G K S
$G\alpha_{i3}$	39-46:	L G A G E S G K S
$G\alpha_{o1}$	39-46:	L G A G E S G K S
$G\alpha_{o2}$	39-46:	L G A G E S G K S
$G\alpha_{t1}$	35-42:	L G A G E S G K S
$G\alpha_{t2}$	39-46:	L G A G E S G K S
$G\alpha_{gust}$	39-46:	L G A G E S G K S
$G\alpha_s$	46-53:	L G A G E S G K S
$G\alpha_q$	45-52	L G **T** G E S G K S
$G\alpha_{12}$	63-70:	L G A G E S G K S

Panel B:

$G\alpha_z$	348-355:	N L K Y **I** G L C
$G\alpha_{i1}$	347-354:	N L K D C G L F
$G\alpha_{i2}$	348-355:	N L K D C G L F
$G\alpha_{i3}$	347-354:	N L K E C G L Y
$G\alpha_{o1}$	347-354:	N L R G C G L Y
$G\alpha_{o2}$	347-354:	N L R G C G L Y
$G\alpha_{t1}$	343-350:	N L K D C G L F
$G\alpha_{t2}$	347-354:	N L K D C G L F
$G\alpha_{gust}$	347-354:	N L K D C G L F
$sG\alpha_{i2}$	359-366:	P L S S **D** S V P
$G\alpha_s$	387-394:	H L R Q **Y** E L L
$G\alpha_q$	352-359:	N L K E **Y** N L V
$G\alpha_{12}$	374-381:	N L K D **I** M L Q

Figure 1.1: Comparison of the amino acid sequence of $G\alpha_z$ with other $G\alpha_i$ family members, and with representative members of the $G\alpha_s$, $G\alpha_q$ and $G\alpha_{12}$ families, in the G1 loop of the GTPase domain (Panel A) and in the carboxyl terminal region (Panel B). $sG\alpha_{i2}$ is an alternative carboxyl terminal spliced variant of $G\alpha_{i2}$ and its predicted amino acid sequence can be found in Montmayeur & Borrelli (1994). The gene bank accession numbers of the other depicted sequences are: for $G\alpha_z$ (XP_109580), $G\alpha_{i1}$ (NP_002060), $G\alpha_{i2}$ (P08752), $G\alpha_{i3}$ (XP_010738), $G\alpha_{o1}$ (AAM12608), $G\alpha_{o2}$ (AAM12609), $G\alpha_{t1}$ (XP_017793), $G\alpha_{t2}$ (NP_005263), $G\alpha_{gust}$ (S24352), $G\alpha_s$ (P04894), $G\alpha_q$ (XP_123396), $G\alpha_{12}$ (Q03113).

its unique amino acid sequence have been confirmed. When the intrinsic rate of GTP hydrolysis of recombinant $G\alpha_z$ is measured, the obtained k_{cat} of 0.05 min^{-1} (at 30°C) is two hundred times slower than the corresponding k_{cat} of $G\alpha_s$, $G\alpha_o$ and the $G\alpha_i$s, which have k_{cat} values in the range of 10 min^{-1} at 30°C (Casey et al., 1990; Fields, 1998). Hence, it would take 10-12 minutes for half of all the bound GTP on $G\alpha_z$ to hydrolyse compared to just several seconds for these other Gα subunits (Casey et al., 1990). Coincidentally, $G\alpha_q$, which has the same threonine substitution as $G\alpha_z$ at position 47 of the protein (Figure 1.1, panel A) also demonstrates a slow k_{cat} of 0.8 min^{-1} (Berstein et al., 1992). Other studies suggest that the substitution with serine at position 42 for the conserved glycine may also contribute to the weak hydrolytic activity of $G\alpha_z$ (Sprang, 1997a). On the other hand, $G\alpha_{12}$, in spite of sharing the same GAGES motif as most other Gα subunits (Figure 1.1, Panel A), also exhibits a very slow GTP hydrolysis rate (k_{cat} of 0.1-0.2 min^{-1}) (Kozasa and Gilman, 1995). Therefore, the intrinsic GTPase activities of G protein α subunits are not determined solely by the GAGES sequence (Fields and Casey, 1997). There are other regions of the protein that contribute as well (Sprang, 1997a).

The sluggish GTPase activity of $G\alpha_z$ means that once $G\alpha_z$ becomes activated, it can remain in that state for a relatively long time. This unusual property of $G\alpha_z$ makes it a possible candidate for mediating signal transduction over long distances. Intriguingly, $G\alpha_z$ has been observed to be transported along the axons of sensory neurons, which led to the hypothesis that $G\alpha_z$ may function as a stable retrograde messenger, carrying information from the axon terminal back to the cell body (Hendry et al., 1995b).

The slow GTP hydrolysis rate of $G\alpha_z$ has also inspired a search for molecules that can stimulate its sluggish GTPase activity (Fields, 1998; Wang et al., 1997). This effort resulted in the identification of a $G\alpha_z$ selective RGS protein, RGSZ1. Like other RGS proteins which can serve as GAPs for Gα subunits, RGSZ1 is a GAP for $G\alpha_z$ (Glick et al., 1998; Wang et al., 1998). According to one estimate, RGSZ1 increases the rate of GTP hydrolysis of GTP bound $G\alpha_z$ more than four hundred fold, bringing its deactivation $t_{1/2}$ to just one second at 30°C (Wang et al., 1998). Besides RGSZ1, another relatively selective GAP for $G\alpha_z$ is RET-RGS1, which has also been shown to increase the GTPase activity of $G\alpha_z$ by more than four hundred times (Wang et al., 1998). RGSZ1 and RET-RGS1 are two of several splice variants from the gene RGS20

(Barker et al., 2001). Since $G\alpha_z$ is also expressed in tissues where RGSZ1 and RET-RGS1 are not expressed (Faurobert et al., 1999; Glick et al., 1998; Wang et al., 1998), the different splice variants of RGS20 may together account for multiple $G\alpha_z$ selective GAP activity in various tissues.

A number of other RGS proteins (including RGSZ2, GAIP, RGS4, RGS10) can also stimulate the slow GTP hydrolysis rate of $G\alpha_z$ *in vitro* (Barker et al., 2001; Popov et al., 1997; Tu et al., 1997; Wang et al., 1999). However, these RGS proteins appear to be relatively broad spectrum GAPs for members of the $G\alpha_i$ family (Berman et al., 1996; Popov et al., 1997) (Barker et al., 2001). RGS4 and GAIP are GAPs for $G\alpha_q$ as well (Hepler et al., 1997; Huang et al., 1997b).

1.2.4 Resistance to cholera toxin, pertussis toxin and N-ethylmaleimide

Consistent with expectations from its amino acid sequence, $G\alpha_z$ has been found to be insensitive to treatment with pertussis toxin (Casey et al., 1990) and the sulfhydrl-alkylating agent, N-ethylmaleimide (Sidhu et al., 1998). Both pertussis toxin and N-ethylmaleimide target the unique carboxyl terminal cysteine residue found in most $G\alpha_i$ family members to disrupt receptor-G protein coupling (Shinoda et al., 1990). Besides $G\alpha_z$, there is only one other $G\alpha_i$ family member that lacks this cysteine residue (Figure 1.1, Panel B). This is an alternative carboxyl terminal spliced form of $G\alpha_{i2}$, which has also been predicted to be insensitive to pertussis toxin treatment (Montmayeur and Borrelli, 1994). However, unlike $G\alpha_z$, which localizes to the plasma membrane (Morales et al., 1998), this spliced $G\alpha_{i2}$ variant resides in the Golgi apparatus, where it has been suggested to be involved in transporting proteins to the endoplasmic reticulum (Picetti and Borrelli, 2000). Therefore, $G\alpha_z$ is currently the only member within the $G\alpha_i$ family, which can mediate pertussis toxin/ N-ethylmaleimide resistant signalling across the plasma membrane.

Like most $G\alpha_i$ family members, $G\alpha_z$ is also insensitive to cholera toxin (Casey et al., 1990). Cholera toxin catalyses the ADP-ribosylation of an arginine residue in the GTPase domain of transducin (Van Dop et al., 1984) and $G\alpha_s$ (Bourne et al., 1989), leading to an inhibition of their GTPase activities (Kaziro et al., 1991). Although this

arginine residue is present in all currently known Gα subunits, only members of the $G\alpha_s$ family and transducin are substrates of the toxin (Hepler and Gilman, 1992; Klemke et al., 2000). This suggests amino acid residues that are not directly modified by the toxin also contribute to determining substrate suitability.

1.2.5 Sensitivity of the spontaneous guanine nucleotide exchange rate of $G\alpha_z$ to Magnesium

Another unique biochemical property of $G\alpha_z$ is the way its spontaneous guanine nucleotide exchange rate is affected by magnesium. All G protein α subunits possess a magnesium binding site that is tightly coupled to the guanine nucleotide binding site (Sprang, 1997a), and the bound magnesium can influence the affinity of Gα for GDP/GTP, and thereby change the spontaneous guanine nucleotide exchange rate on the α subunit (Higashijima et al., 1987). The basal guanine nucleotide exchange rate of $G\alpha_z$ has been determined and found to be optimal at low micromolar concentrations of free magnesium ($k= 0.02min^{-1}$ at 30°C, 0.5μM Mg^{2+}) (Casey et al., 1990). This result stands in contrast to other Gαs where the optimum rate of guanine nucleotide exchange occurs at significantly higher millimolar concentrations of magnesium (Higashijima et al., 1987; Kozasa and Gilman, 1995; Linder et al., 1990; Singer et al., 1994). In the case of $G\alpha_z$, magnesium concentrations exceeding 50μM cause its rate of guanine nucleotide exchange to decline rapidly, reaching a value close to zero as magnesium levels rise to millimolar concentrations (Casey et al., 1990). This phenomenon is explained by stabilization of the GDP-bound form of $G\alpha_z$ by magnesium (Casey et al., 1990). In contrast, magnesium has virtually no effect on the affinities of other Gαs for GDP, but helps to stabilize their GTP (or GTPγS) bound conformation instead (Gilman, 1987; Graziano et al., 1989; Higashijima et al., 1987).

The intracellular free magnesium concentration is typically in the range of 0.5-1 mM in neurons and probably most other cells (Li et al., 2001; McCarthy and Kumar, 1999; Rajdev and Reynolds, 1995). At these magnesium concentrations, there would practically be no dissociation of GDP from $G\alpha_z$, and activation of $G\alpha_z$ would be expected to show high fidelity, whereby only a receptor capable of coupling to $G\alpha_z$ could promote the exchange of GDP for GTP (Barr et al., 1997).

1.2.6 Sensitivity of $G\alpha_z$ selective GAPs to Magnesium

In contrast to the effects of magnesium on the spontaneous guanine nucleotide exchange reaction of $G\alpha_z$, the slothful GTP hydrolysis rate of activated $G\alpha_z$ is relatively unaffected by the divalent cation (Casey et al., 1990). Due to the sluggishness of $G\alpha_z$'s intrinsic GTPase activity, hydrolysis of the bound GTP on $G\alpha_z$ is expected to be facilitated by a RGS protein *in vivo*. Interestingly, while the GAP activities of non-selective RGS proteins (RGS4 and RGS10) show a slight decline as the magnesium concentration rises from 1μM to 1mM, the GAP activities of both $G\alpha_z$ specific GAPs, RGSZ1 and RET-RGS1, increase four fold over this magnesium concentration range (Wang et al., 1998). Therefore, the association of these $G\alpha_z$ specific GAPs with $G\alpha_z$ would lead to further dampening of $G\alpha_z$ signalling by ensuring rapid signal termination under normal physiological conditions.

1.2.7 Inactivation of $G\alpha_z$ by arachidonic acid

Besides magnesium, arachidonic acid and other cis-unsaturated fatty acids have also been shown to inhibit the guanine nucleotide exchange reaction of $G\alpha_z$ (Glick et al., 1996). The inhibition occurs after GDP has dissociated from $G\alpha_z$, through a direct interaction of the lipid micelle with the nucleotide free form of the protein. This prevents $G\alpha_z$ from binding GTP, leading to inactivation of the protein (Glick et al., 1996). The effect of arachidonic acid appears specific for $G\alpha_z$, as guanine nucleotide binding to $G\alpha_{i1}$, $G\alpha_o$ and $G\alpha_s$ are not affected, and $G\alpha_{i3}$ is inhibited to a significantly smaller degree (Glick et al., 1996). In the cell, arachidonic and other fatty acids are synthesized from membrane lipids through activation of the phospholipase A2 enzyme (Alberts et al., 1994; Farooqui et al., 2000). Stimulation of a number of receptor systems, including serotonin (Felder et al., 1990), glutamate (Pellerin and Wolfe, 1991), acetylcholine (Tence et al., 1994) and dopamine (Vial and Piomelli, 1995), can elicit arachidonic acid release. However, whether the ensuing transient increase in arachidonic acid concentration near the plasma membrane has an effect on $G\alpha_z$, and the physiological relevance of $G\alpha_z$ inactivation by such a mechanism (if it occurs *in vivo*) is currently unknown.

1.2.8 Lipid modification of $G\alpha_z$

At a lower concentration, arachidonic acid may have other effects on $G\alpha_z$. In blood platelets, arachidonic acid has been reported to bind $G\alpha_z$ via a reversible thioester bond (Hallak et al., 1994b). The function of the arachidonoylation is currently unknown but such lipid modification is expected to increase the hydrophobicity of $G\alpha_z$, strengthening its association with the membrane compartment. Besides arachidonoylation, $G\alpha_z$ is also subjected to myristoylation (Mumby et al., 1990) and palmitoylation (Morales et al., 1998) due to the presence of a MGCXXS sequence at its amino terminal (Chen and Manning, 2001). The second glycine residue is myristoylated while the third cysteine residue is reversibly palmitoylated, with myristoylation as a pre-requisite for palmitoylation to occur (Hallak et al., 1994a). Myristoylation increases the hydrophobicity of $G\alpha_z$ and is essential for anchoring $G\alpha_z$ to membranous compartments (Hallak et al., 1994a). However, the targeting of $G\alpha_z$ to the plasma membrane appears to be controlled by its association with the prenylated $\beta\gamma$ subunits (Fishburn et al., 2000).

Palmitoylation of $G\alpha_z$ has been found to inhibit the GAP activity of GAIP, RGS4, RGS10 and brain $G\alpha_z$-GAP (which consists of RGSZ1 and possibly other $G\alpha_z$ GAPs) towards $G\alpha_z$, and de-palmitoylation reversed this inhibition (Tu et al., 1997). Given $G\alpha_z$ has a very slow intrinsic GTPase activity (Casey et al., 1990), the association of $G\alpha_z$ with a GAP is almost necessary for signal termination. Reversible palmitoylation may therefore represent a very important mechanism for regulating $G\alpha_z$ signalling.

1.2.9 Phosphorylation of $G\alpha_z$

One other important biochemical property of $G\alpha_z$ is its ability to be phosphorylated. $G\alpha_z$ is phosphorylated by both protein kinase C (Kozasa and Gilman, 1996; Lounsbury et al., 1991) and p21-activated kinase 1 (PAK1) (Wang et al., 1999). The phosphorylation by protein kinase C occurs primarily on two amino terminal serine residues, serine 16 and serine 27 (Lounsbury et al., 1993), with serine 27 being the

kinetically preferred phosphorylation site (Wang et al., 1999). PAK1, on the other hand, phosphorylates $G\alpha_z$ specifically at serine 16 only (Wang et al., 1999). The phosphorylation by both kinases takes place independently of the activation state of $G\alpha_z$ and causes a decrease in affinity of the α subunit for the $\beta\gamma$ subunit (Fields and Casey, 1995; Wang et al., 1999). Phosphorylation of $G\alpha_z$ also blocks the GAP activities of all $G\alpha_z$-GAPs that are studied, including RGSZ1, GAIP, RET-RGS1, RGS4 and brain $G\alpha_z$ GAP (Glick et al., 1998; Wang et al., 1998; Wang et al., 1999). Accordingly, if $G\alpha_z$ is already in its dissociated activated form when it is being phosphorylated, it will likely remain in that state for a much longer period of time due to the significant attenuation of $G\alpha_z$-GAP activity. If G_z is as a heterotrimer when $G\alpha_z$ is being phosphorylated, the consequent decrease in affinity of $G\alpha_z$ for the $\beta\gamma$ subunits (as a result of the phosphorylation) may help G_z to become activated more easily since the $\beta\gamma$ dimer (as a GDI) normally inhibits GDP dissociation from the α subunit (Sprang, 1997b). However, one may expect the dissociated GTP-bound form of $G\alpha_z$ to be the preferred phosphorylation substrate of both protein kinase C and PAK1 as the presence of the $\beta\gamma$ subunit inhibits phosphorylation of $G\alpha_z$ by both kinases (Fields and Casey, 1995; Kozasa and Gilman, 1996; Wang et al., 1999). Therefore, signalling events that lead to the activation of either PAK1 or protein kinase C are likely to exert a significant positive impact on $G\alpha_z$ signalling.

1.3 Tissue distribution of $G\alpha_z$

The tissue distribution of a protein can sometimes provide important clues about its function. For instance, gustducin, a G protein found almost exclusively in taste buds (McLaughlin et al., 1992), is involved in the signal transduction of bitterness (Margolskee, 2002). $G\alpha_z$ also has a rather restrictive tissue distribution, suggesting it may have a special role in those cells that express the protein.

Within the brain, $G\alpha_z$ mRNA has been consistently detected in the cerebral cortex, amygdala, caudate nucleus, putamen, nucleus accumbens, substantia nigra, subthalamic nucleus, thalamus, cerebellum, medulla and hippocampus (Friberg et al., 1998; Glick et al., 1998; Wang et al., 1998). $G\alpha_z$ mRNA is either present at very low levels or is absent in the globus pallidus (Friberg et al., 1998), and is found variably in the corpus

callosum (Friberg et al., 1998; Glick et al., 1998; Wang et al., 1998). Immunohistochemical localization has confirmed the existence of $G\alpha_z$ in the cerebral cortex, hippocampus (where neurons of the dentate gyrus and Ammon's horn stain intensely), basal ganglia, thalamus and cerebellum, and absence in glia and white matter tracts (Hinton et al., 1990). $G\alpha_z$ has also been localized by both in situ hybridization and western blot techniques to the hypothalamus (Sanchez-Blazquez et al., 1995; Serres et al., 2000), and has been reported to be present in the mouse periaqueductal gray (Garzon et al., 1997b; Garzon et al., 1997a; Sanchez-Blazquez et al., 1995). Elsewhere in the nervous system, $G\alpha_z$ has been found in the pituitary gland (Paulssen et al., 1991), the retina (Fong et al., 1988; Hinton et al., 1990; Jiang et al., 1991; Zigman et al., 1994), the ear (Magovcevic et al., 1995) and in sensory and sympathetic ganglia (Hendry et al., 1995b; Hinton et al., 1990; Kelleher et al., 1998) of the spinal cord (Wang et al., 1998). In short, $G\alpha_z$ appears to be ubiquitous within much of the nervous system.

Among peripheral tissues, $G\alpha_z$ is expressed in the adrenal medulla (Casey et al., 1990; Hinton et al., 1990), in blood platelets (Carlson et al., 1989; Casey et al., 1990; Gagnon et al., 1991) (Kelleher et al., 2001), in megakaryocytes (Gagnon et al., 1991), in interleukin 2 activated natural killer cells (Al Aoukaty et al., 1997; Maghazachi et al., 1996), in the islets of the pancreas (Zigman et al., 1994), in sperm cells (Glassner et al., 1991) and at very low levels in erythrocytes (Premont et al., 1989). Using northern or western blot techniques, $G\alpha_z$ has also been detected in tissues such as the heart (Garibay et al., 1991), the liver (Spicher et al., 1988), the kidney (Spicher et al., 1988; Zigman et al., 1994), the lung (Zigman et al., 1994), the spleen (Zigman et al., 1994) and the placenta (el Mabrouk et al., 1996). However, the findings in these areas have not been consistently replicated by others (Casey et al., 1990; Garibay et al., 1991; Zigman et al., 1994), suggesting $G\alpha_z$ is either present at low levels in these tissues or the detection is due to contamination from blood platelets, which appear to be a relatively abundant source of the protein (Casey et al., 1990; Gagnon et al., 1991; Woulfe et al., 2002).

Consistent with the predominant localization of $G\alpha_z$ in neuronal, adrenal, pituitary and blood tissues, $G\alpha_z$ has also been found in a number of cell lines derived from these sources. These include the neuroblastoma cell lines SH-SY5Y (Ammer and Schulz, 1994), SK-N-SH and SH-EP (Garibay et al., 1991), the pheochromocytoma cell line PC12 (Garibay et al., 1991), the mouse anterior pituitary cell line AtT-20 (Hallak et al.,

1994a), the rat pituitary tumor cell lines GH_3, GH_4C_1 and GH_12C_1 (Paulssen et al., 1991), the megakaryoblastic leukemia cell line MEG-01 (Nagata et al., 1995) and the basophilic leukemia cell line RBL-2H3 (Hallak et al., 1994a; Hide et al., 1991). Additionally, $G\alpha_z$ has been reported to be present in the hepatoma cell line, HepG2 (Garibay et al., 1991) and in the African green monkey kidney cell line, COS-7 (Belcheva et al., 2000), suggesting that $G\alpha_z$ may normally also be expressed in liver (Spicher et al., 1988) and kidney tissues (Spicher et al., 1988; Zigman et al., 1994), or the expression occurs as a result of cell transformation.

At the ultrastructural level, $G\alpha_z$ immunoreactivity is associated with the outermost aspect of the nuclear membrane, the endoplasmic reticulum, the inner surface of the plasma membrane, and with small granules on the microtubules of proximal dendrites (Hinton et al., 1990). $G\alpha_z$ also accumulates on both sides of a nerve ligature and this accumulation is sensitive to colchicine, an inhibitor of microtubule function (Crouch et al., 1994). These observations are consistent with $G\alpha_z$ being transported in neurons, although its physiological significance is unclear.

1.4 Guanine nucleotide exchange factors for $G\alpha_z$

$G\alpha_z$ has a slow basal guanine nucleotide exchange rate ($k= 0.02min^{-1}$ at 30°C and 0.5μM Mg^{2+}) and this rate further declines to near zero at physiological concentrations of magnesium (0.5-1mM) (Casey et al., 1990). This property of $G\alpha_z$ appears to be unique among $G\alpha$ subunits and is similar to that seen with the small GTPase Ras, a protein with oncogenic potential (Hall and Self, 1986). This may suggest that spontaneous activation of $G\alpha_z$, if not carefully regulated, can lead to disastrous consequences. Therefore, G_z activation is expected to show high fidelity, requiring a GEF to catalyse the exchange of GDP for GTP on the α subunit.

1.4.1 G protein coupled receptors

GPCRs are the de facto GEFs for G proteins. They comprise one of the largest and most diverse superfamily of proteins in our body (Bockaert et al., 2002). Based on

hydropathy analysis of their amino acid sequences, all GPCRs are predicted to share a common three dimensional architecture, characterized by seven hydrophobic trans-membrane helices (TM-I to TM-VII) connected by six alternating extracellular (E-1 to E-3) and intracellular loops (I-1 to I-3) (Bockaert and Pin, 1999)) (Harmar, 2001). Additionally, a fourth intracellular loop (I-4) linking the seventh transmembrane helix to palmitoylated cysteine residues at the carboxyl terminal is found in some members of the superfamily (Marin et al., 2000). The extracellular surface, containing the amino-terminal and extracellular loops, is responsible for ligand discrimination and binding (Schwartz, 1994). The cytoplasmic face, which contains the intracellular loops and the carboxyl terminal tail, is involved in the recognition and activation of G proteins (Conklin and Bourne, 1993). The molecular mechanism by which ligand binding causes activation of a G protein is still unknown (Hamm, 2001). Precise mechanistic details may vary between members of this diverse superfamily and may depend on the particular G protein a receptor is coupled to (Bae et al., 1997; Cabrera-Vera et al., 2002). However, it is generally possible to subdivide the superfamily of GPCRs based on their preference for members of a particular G protein α subunit family (Hur and Kim, 2002). For instance, opioid receptors couple preferentially to members of the $G_{i/o}$ family (Standifer and Pasternak, 1997) while vasopressin V1 receptors predominantly couple to members of the $G_{q/11}$ family (Birnbaumer, 2000). This observation highlights the existence of interaction sites on both receptors and G protein α subunits for directing specificity of coupling (Moller et al., 2001), and suggests homologous G protein α subunits from the same G protein family may share certain common structural features that allow them to interact with a particular receptor.

Accordingly, G_z, a member of the $G_{i/o}$ family, has been found to be capable of coupling to all $G_{i/o}$ coupled receptors in transfection and reconstitution studies (Ho and Wong, 1998). These receptors include the serotonin 1A receptor (Barr et al., 1997; Butkerait et al., 1995), the μ (Chan et al., 1995), δ (Tsu et al., 1995a) and κ (Lai et al., 1995) opioid receptors, the nociceptin (orphanin FQ) receptor (Chan et al., 1998), the neurokinin 1 receptor (Barr et al., 1997), the thrombin receptor (Barr et al., 1997), the adenosine A1 receptor (Wong et al., 1992), the α_2 adrenergic receptor (Wong et al., 1992), the muscarinic M2 receptor (Parker et al., 1991), the melatonin 1a, 1b and 1c receptors (Ho and Wong, 1998), the lysophosphatidic acid receptor (Wong et al., 1992), the complement C5a receptor (Shum et al., 1995), the formyl peptide receptor (Tsu et al.,

1995b), the dopamine D5 receptor (Sidhu et al., 1998) and all members of the dopamine D2-like receptor family (Obadiah et al., 1999).

These *in vitro* studies indicate G_z possess the necessary structural elements required for recognition by these receptors. However, it does not tell us whether these receptor-G_z interactions occur *in vivo*, and if it does, in which specific tissues there is actual receptor coupling with G_z. The functional coupling to G_z *in vivo* is determined by a variety of factors including the level of expression of G_z at various points in time, the relative abundance of G_z compared to other $G_{i/o}$ family members in regions of the cell where receptors are found (e.g. axon terminal versus cell body), as well as the specific combinations of β and γ subunits available for associating with $G\alpha_z$ to couple to the receptor. Given that $G\alpha_{i1}$, $G\alpha_{i2}$ and $G\alpha_{i3}$ are widely expressed (Hepler and Gilman, 1992) and are present at high levels in brain cells (Sternweis and Robishaw, 1984) and in blood platelets (Woulfe et al., 2002) (the principal tissues where $G\alpha_z$ have been found) and $G\alpha_o$, another $G\alpha_i$ family member, is the major G protein α subunit in neurons, accounting for 0.5% of all brain membrane proteins (Asano et al., 1988), the capacity of G_z to compete with these other abundant $G_{i/o}$ family members for preferential coupling to a receptor, will depend on its relative affinity for the receptor, the comparative G_z concentration in the local region of the cell where the receptor is found and whether special scaffolding proteins exist that interact preferentially with G_z for organization of the receptor G protein signalling complex (Albert and Robillard, 2002; Huang et al., 1997a; Milligan and White, 2001). Additionally, the composition of the β and γ subunits that $G\alpha_z$ binds to *in vivo* is also likely to influence the interaction of G_z with a receptor. It has been found that the receptor interacting domains of G proteins are not only on the α subunit (Cabrera-Vera et al., 2002), but also incorporate the carboxyl terminal of the γ subunit (Azpiazu and Gautam, 2001; Wess, 1998), and possibly the β propeller region of the β subunit (Taylor et al., 1996). Consistent with the involvement of the βγ dimer in receptor interaction, the identity of the β subunit (McIntire et al., 2001) and the γ subunit (Hou et al., 2000) have been found to directly influence the efficiency of G protein coupling. The composition of the βγ dimers that partner with $G\alpha_z$ has never been investigated, and it is also not known whether different members of the $G\alpha_i$ family vary in their preference for particular βγ subunits. This is an important area of research that will further our understanding of the basis of receptor–G protein coupling.

To date, a number of *in vivo* studies have investigated the receptors that couple to G_z in different body tissues. In mouse periaqueductal gray, an area of the brain involved in the analgesic effects of opioids and the processing of nociceptive information (Smith et al., 1988; Sohn et al., 2000), the μ opioid receptors were found to couple to both G_{i2} and G_z (Garzon et al., 1997b; Garzon et al., 1997a). This was shown by the ability of antibodies against $G\alpha_{i2}$ and $G\alpha_z$ to block morphine and [D-Ala^2,N-MePhe^4,Gly-ol^5] enkephalin (DAMGO) stimulated low K_m GTPase activities of the two G proteins (Garzon et al., 1997b). Chronic treatment with antisense oligonucleotides to $G\alpha_z$ produced the same effect as $G\alpha_z$ antibodies. Although morphine and DAMGO were preferential agonists at the μ receptor, they can also bind δ and κ opioid receptors with significantly lower affinities (Goldstein and Naidu, 1989). The authors demonstrated that the effects of morphine and DAMGO in their experiments were due almost entirely to the μ receptor as the δ receptor antagonist ICI-174,864 had negligible effects on morphine and DAMGO stimulated low K_m GTPase activities (Garzon et al., 1997b). In contrast, antibodies and oligonucleotides against $G\alpha_z$ had no effect on the low K_m GTPase activity stimulated by the δ receptor agonist [D-Pen^{2,5}]enkephalin (DPDPE), while antibodies against $G\alpha_{i2}$ produced significant effects (Garzon et al., 1997b). The results of this experiment suggest that μ opioid receptors in the mouse periaqueductal gray are coupled to both G_{i2} and G_z, while δ opioid receptors are coupled to G_{i2}. In another study using immunoelectrophoresis to examine the activation of Gα subunits from mouse periaqueductal gray membranes as a result of receptor stimulation, morphine and DAMGO were found to cause a dose dependent increase in $G\alpha_{i2}$ and $G\alpha_z$ associated immunoreactivities (Garzon et al., 1997a). This morphine and DAMGO induced activation of $G\alpha_{i2}$ and $G\alpha_z$ was not significantly affected by the δ receptor antagonist ICI-174,864 (Garzon et al., 1997a), again indicating that stimulation of the μ receptor by both agonists is responsible for the effects. In this experiment, the δ receptor agonist DPDPE also caused activation of $G\alpha_{i2}$, but not $G\alpha_z$. In comparison, a second δ receptor agonist, [D-Ala^2]deltorphin II, stimulated both $G\alpha_{i2}$ and $G\alpha_z$, although the stimulation was significantly greater for $G\alpha_{i2}$ than for $G\alpha_z$ (Garzon et al., 1997a). Another interesting finding from this experiment is that of the two μ receptor agonists, morphine is more effective than DAMGO in the stimulation of $G\alpha_z$ (ED_{50} for morphine = 0.17± 0.01pM, ED_{50} for DAMGO = 0.36± 0.05nM), while the reverse is true for the stimulation of $G\alpha_{i2}$ (ED_{50} for morphine = 0.80± 0.06nM, ED_{50} for DAMGO

= 16.0± 1.4pM) (Garzon et al., 1997a). One possible explanation for this intriguing phenomenon is that the binding of morphine and DAMGO to the μ receptor may result in the selective stabilization of different receptor conformational states, which vary in their preference for coupling to different G proteins (Kenakin, 1997). Consistent with coupling of the μ opioid receptors to G_z and G_{i2}, the same group of researchers have gone on to demonstrate that intracerebroventricular (i.c.v.) administration of antibodies or antisense oligonucleotides against either $G\alpha_{i2}$ or $G\alpha_z$, led to an impairment of both morphine and DAMGO analgesia (Sanchez-Blazquez et al., 1993; Sanchez-Blazquez et al., 1995). To verify their technique, they also demonstrated that their antisense treatment reduced $G\alpha_z$ protein expression in the periaqueductal gray, striatum, thalamus and hypothalamus by between 16% to 54% (Sanchez-Blazquez et al., 1995). As for analgesia mediated by the δ receptor agonists, DPDPE and [D-Ala^2]deltorphin II, antisense oligonucleotides and antibodies against $G\alpha_z$ have no effect while those against $G\alpha_{i2}$ and $G\alpha_{i3}$, caused a reduction in analgesia (Sanchez-Blazquez et al., 1993; Sanchez-Blazquez et al., 1995). This behavioural finding is consistent with preferential coupling of δ opioid receptors to G_{i2} and G_{i3}. A similar study conducted by a different group of researchers, however, showed that while i.c.v. administration of $G\alpha_{i2}$ antisense oligonucleotides significantly reduced morphine analgesia, antisense oligonucleotides against $G\alpha_z$ have very little effect (Standifer et al., 1996). The later researchers, nonetheless, did not validate whether their $G\alpha_z$ antisense, which was made against the human $G\alpha_z$ gene sequence, was effective in decreasing $G\alpha_z$ protein expression in mice. A blast search (Altschul et al., 1997) based on the human $G\alpha_z$ sequence that was employed, fail to recognize the mouse $G\alpha_z$ mRNA sequence. This would explain the western blot data obtained by a second group of investigators, who employed an identical human $G\alpha_z$ antisense, and found very little attenuation of mouse $G\alpha_z$ protein expression even after a large quantity (20μg) of oligonucleotides had been injected (Karim and Roerig, 2000).

In blood platelets, the α_{2a} adrenergic receptor has been shown to couple preferentially to G_z, despite G_{i2} being more highly expressed (Woulfe et al., 2002; Yang et al., 2000). Epinephrine, the natural ligand of adrenergic receptors, inhibits cAMP formation and activates the small GTPase Rap1 in platelets, both of which may contribute to platelet activation (Woulfe et al., 2002; Yang et al., 2000). In platelets that lack $G\alpha_z$, this epinephrine induced inhibition of cAMP production and Rap1 activation is diminished

significantly (Woulfe et al., 2002; Yang et al., 2000). Furthermore, epinephrine lost its ability to potentiate platelet aggregation elicited by a number of platelet activators including ADP, serotonin, collagen, GYPGQV (a PAR4 thrombin receptor agonist) and U46619 (a thromboxane A_2 receptor agonist) in $G\alpha_z$ deficient platelets (Yang et al., 2000). In congruence with this, $G\alpha_z$ deficient mice also showed a reduction in thromboembolic mortality caused by epinephrine and collagen, although the mortality rate following ADP (which activates a $G\alpha_{i2}$ coupled receptor) (Jantzen et al., 2001) and collagen administration were the same (Yang et al., 2000). These results clearly demonstrate that in the absence of $G\alpha_z$, epinephrine responses are impaired and suggest α_{2a} adrenergic receptors in platelets couple preferentially to G_z.

In the nervous system, α_{2a} adrenergic receptors are involved in the modulation of many physiological and behavioural processes including pain, mood, anesthesia, vigilance and body temperature (Kable et al., 2000; Lakhlani et al., 1997; Schramm et al., 2001). The effects of mood elevating drugs in $G\alpha_z$ deficient mice have been investigated. Compared to wildtype controls, mice deficient in $G\alpha_z$ failed to respond to the anti-depressant effects of desipramine and reboxetene, two compounds that block neurotransmitter reuptake at norepinephrine transporters (Yang et al., 2000). This phenotype of $G\alpha_z$ deficient mice bears superficial resemblance to that of the α_{2a} adrenergic receptor knockout mice, which are insensitive to the antidepressant effects of imipramine, a norepinephrine and serotonin reuptake inhibitor (Schramm et al., 2001). These preliminary findings are consistent with a possible coupling of G_z to α_{2a} adrenergic receptors in the nervous system.

The $G\alpha_z$ deficient mouse demonstrates another interesting phenotype: an enhanced locomotor response to cocaine. This increase in cocaine induced locomotor activity exhibited by $G\alpha_z$ knockout mice is sustained over the entire period of cocaine treatment (Yang et al., 2000). Although cocaine inhibits reuptake by the norepinephrine transporter, it also blocks serotonin and dopamine transporters (Uhl et al., 2002). Studies on mice that lack either the norepinephrine, serotonin or dopamine transporters have shown the dopaminergic system to be responsible for the locomotor stimulation produced by cocaine (Uhl et al., 2002). The locomotor response to cocaine is abolished in mice that lack the dopamine transporter (Sora et al., 1998; Sora et al., 2001b), while still present in mice that are deficient in either the serotonin or norepinephrine

transporters (Sora et al., 2001b; Xu et al., 2000a). The locomotor hyperactivity observed in $G\alpha_z$ deficient mice following cocaine treatment might therefore suggest an alteration in dopaminergic neurotransmission in the mutant mice and G_z could be coupled to a dopamine receptor. Experiments *in vitro* have established G_z to be capable of transducing the signal produced by activation of dopamine D2, D3 and D4 receptors (Obadiah et al., 1999). There is also evidence that the dopamine D5 receptor can couple to G_z (Sidhu et al., 1998). Dopamine receptors are localized pre and post-synaptically (Missale et al., 1998; Picetti et al., 1997; Rivera et al., 2002; Svingos et al., 2000). Presynaptic receptors consist of two types: autoreceptors, which are found on dopaminergic neurons and function to regulate dopamine release; and heteroreceptors, which control the release of other neurotransmitters. Studies of dopamine D2 and D3 receptor knockout mice have established autoreceptors to be primarily of the D2 type (Benoit-Marand et al., 2001; L'hirondel et al., 1998; Rouge-Pont et al., 2002; Schmitz et al., 2001), with the D3 receptor making only a minor contribution (Joseph et al., 2002). Presynaptic D4 and D5 receptors, on the other hand, appear to be heteroreceptors, where there is evidence that they regulate glutamate and acetylcholine release respectively (Hersi et al., 2000; Price and Pittman, 2001; Rubinstein et al., 2001). The absence of dopamine D2 receptors in mice has been found to produce an augmented cocaine induced increase in extracellular dopamine concentration, which has been attributed to an impairment of D2 autoreceptor function (Rouge-Pont et al., 2002). Cocaine elicited dopamine release is an important contributor to cocaine induced hyperlocomotor activity since the response can be blocked by dopamine receptor antagonists (Delfs et al., 1990) (Neisewander et al., 1995). Therefore, it is possible that cocaine's increased locomotor activation in $G\alpha_z$ knockout mice may be explained by the coupling of G_z to a dopamine autoreceptor, whose function is impaired as a result of the loss of $G\alpha_z$.

In the hypothalamus, cells in the paraventricular nucleus (PVN) have been shown to contain $G\alpha_z$ (Serres et al., 2000). These PVN neurons also express serotonin (5HT) 1A receptors (Li et al., 1997), and stimulation of these receptors results in the secretion of oxytocin and corticotropin releasing hormone (CRH) (Bagdy, 1996) from magnocellular and parvocellular cells respectively (Li et al., 1997). From the hypothalamus, the CRH secreted is released into the pituitary portal blood, which in turn stimulate corticotropes in the anterior pituitary gland to release adrenocorticotropic hormone (ACTH) (Aguilera et al., 2001). The reduction of $G\alpha_z$ levels in the PVN through the infusion of $G\alpha_z$ antisense into the ventricles, has been shown to attenuate the rise in plasma oxytocin

and ACTH caused by activation of 5HT-1A receptors (Serres et al., 2000). It was further demonstrated that this 5HT-1A receptor stimulated oxytocin secretion was resistant to pertussis toxin, while the ACTH secretion was partially toxin sensitive, consistent with the involvement of a pertussis toxin resistant G protein in the transduction pathway (Serres et al., 2000). Based on these results, 5HT-1A receptors in the PVN have been proposed to be preferentially coupled to G_z (Serres et al., 2000). While this may be the case, it is also possible that the administration of $G\alpha_z$ antisense into the ventricles, has disrupted G_z coupling to other receptors, which are in the circuitry that produced the 5HT-1A receptor mediated responses. This is particularly relevant to the 5HT-1A receptor stimulated ACTH secretion, which is dependent on the integrity of CRH type I receptors on corticotropes (Aguilera et al., 2001).

There are two major CRH receptors in the brain, the type I and type II receptors, each with their own alternate spliced variants (Perrin and Vale, 1999). CRH type I receptors are localized predominantly in the pituitary where they mediate ACTH secretion (Aguilera et al., 2001), as well as in the olfactory bulb, cerebral cortex, cerebellum, thalamus, hypothalamus and brain stem (Radulovic et al., 1998). The type II receptors, on the other hand, are found mainly in frontal and occipital cortices, amygdala, lateral septum, entorhinal cortex and hypothalamic regions (Primus et al., 1997). CRH binds with higher affinity to the type I receptor (Primus et al., 1997), while urocortin is the predominant ligand at the type II receptor based on its anatomical localization (Kozicz et al., 1998). Both CRH type I and II receptors are believed to preferentially couple to G_s to activate adenylate cyclase activity (Chen et al., 1993; Lovenberg et al., 1995). However, in cerebral cortical membrane preparations, CRH and urocortin were found to stimulate the incorporation of [α-^{32}P]-GTP-γ-azidoanlide, a photoreactive form of GTP into $G\alpha_s$, $G\alpha_i$, $G\alpha_{q/11}$, $G\alpha_o$ and $G\alpha_z$, suggesting that CRH receptors can couple to all of these G proteins (Grammatopoulos et al., 2001). The G protein stimulation profiles of the two peptide agonists were similar, with the exception of $G\alpha_{q/11}$, which was stimulated to a greater extent by urocortin (Grammatopoulos et al., 2001). One common criticism of the [α-^{32}P]-GTP-γ-azidoanlide labeling technique is that the labeling of a large number of G protein α subunits by receptor stimulation could be an experimental artifact in broken cell preparations, where membrane disruptions allow the formation of unnatural receptor G protein complexes (Albert and Robillard, 2002). To address this and other possible concerns, the authors demonstrated that under their experimental conditions, stimulation of the α1 adrenergic receptor labeled only $G\alpha_q$,

stimulation of the α2 receptor labeled only $G\alpha_{i/o}$ and stimulation of the β adrenergic receptor labeled only $G\alpha_s$, which are consistent with the preferred G protein coupling of these receptors discovered using other techniques (Grammatopoulos et al., 2001). From these results, it is possible that G_z may be coupled to a receptor stimulated by CRH and urocortin in the cerebral cortex. Interestingly, CRH receptors in rat Leydig cells has been reported to couple to a pertussis toxin resistant G protein to inhibit adenylate cyclase activity (Ulisse et al., 1989; Ulisse et al., 1990). To date, $G\alpha_z$ has not been reported to be present in Leydig cells, although it has been found in sperm cells (Glassner et al., 1991).

Chemokine receptors are classified into four subfamilies and named according to the type of chemokines they bind to. These are (i) the CC receptor subfamily (with eleven members from CCR1 to CCR11), which binds ligands CCL1 to CCL27, (ii) the CXC receptor subfamily (with five members from CXCR1 to CXCR5), which binds CXCL1 to CXCL14, (iii) the CX3C receptor subfamily (with a single member CX3CR1) that binds CX3CL1 and (iv) the XC receptor subfamily (with a single member XC-R1) that binds XCL1 and XCL2 (Bajetto et al., 2001). The majority of ligands in the CC and CXC receptor families are promiscuous and can bind more than one receptor (Bajetto et al., 2001). Interleukin-2 activated natural killer (IANK) cells express $G\alpha_z$ (Al Aoukaty et al., 1997; Maghazachi et al., 1996) and the chemotaxis of these cells in response to CCL2 (monocyte chemoattractant protein 1; MCP-1), CCL3 (macrophage inflammatory protein 1α, MIP-1α) and CCL5 (regulated on activation normal T cell-expressed and secreted; RANTES) were blocked by antibodies against $G\alpha_s$, $G\alpha_o$ and $G\alpha_z$ (Al Aoukaty et al., 1996). The antibodies also prevented agonist stimulated binding of the non-hydrolysable GTP analogue, GTPγS, as well as GTPase activities elicited by these agonists (Al Aoukaty et al., 1996). Being promiscuous, the three ligands can potentially bind to a number of CC receptors, including CCR1, CCR2, CCR3 CCR4, CCR5 and CCR11 (Bajetto et al., 2001). CCR5 is not expressed by IANK cells (Inngjerdingen et al., 2001), leaving the five remaining CC receptors as possible candidates for coupling to G_z. Additionally, G_z can also couple to the CCR6 and the CX3CR1 receptors found in IANK cells (Al Aoukaty et al., 1998; Inngjerdingen et al., 2001). This is demonstrated by the capacity of the CCR6 receptor specific agonist, CCL20 (macrophage inflammatory protein 3α; MIP-3α) and CX3CR1 receptor specific agonist CX3CL1 (fractalkine) to stimulate GTPγS binding to $G\alpha_z$ (Al Aoukaty et al., 1998).

However, in the same experiment, CCL20 also activated G_o and G_q, and CX3CL1 stimulated G_i as well (Al Aoukaty et al., 1998), suggesting that the CCR6 and CX3CR1 receptors are capable of coupling to multiple G proteins (inclusive of G_z) or there are as yet undiscovered receptors that the ligands can bind to (that may couple to G_z). Chemokine receptors (e.g. CX3CR1) have also been found in neurons (Bajetto et al., 2001; Meucci et al., 1998), and it is possible that G_z may mediate the functions of some of these receptors in the nervous system.

1.4.2 G protein coupled receptor dimers and oligomers

GPCRs can also associate with one another to form dimers and/or oligomers (Bouvier, 2001; Rios et al., 2001). This is supported by data from radiation inactivation, Western Blot, co-immunoprecipitation and cross-linking experiments, as well as results from bioluminescence and fluorescence resonance energy transfer studies in living cells (Rios et al., 2001; Salahpour et al., 2000). When two GPCRs labeled with different fluorescent molecules are co-expressed in the same cells, they can effect resonance energy transfer from one to the other, indicating that the GPCRs lie very close to one another on the plasma membrane (Angers et al., 2000; McVey et al., 2001; Ramsay et al., 2002). Furthermore, structural data from crystallography of the rhodopsin receptor suggested the cytoplasmic surface of a GPCR monomer is just barely broad enough for interaction with both the α and $\beta\gamma$ subunits of a G protein (Bourne and Meng, 2000). This may imply that one GPCR can only couple to one G protein; or higher order GPCR complexes exist, which may allow simultaneous interactions with more than one G protein, as well as leaving sufficient room for interaction with accessory proteins such as arrestins, G protein coupled receptor kinases and scaffolding proteins. Taken together, the current available evidences suggest that GPCR complexes are likely to exist *in vivo*.

There are two types of GPCR complexes: homomers which are formed from identical GPCR units and heteromers which arise from the association between distinct protein units (Rios et al., 2001). Not all GPCRs can heteromerize with one another. For instance, among the opioid receptors, the δ and κ receptors (Jordan and Devi, 1999), as well as the μ and δ receptors (George et al., 2000; Gomes et al., 2000) can heteromerize with each other, but the μ and κ receptors are not able to (Jordan and Devi, 1999).

The list of GPCRs capable of forming homomers with themselves or heteromers with another GPCR is rapidly expanding, and includes a number of GPCRs previously identified to be capable of coupling to G_z. As current technology does not allow easy determination of receptor structure, whether the receptors that interacted with G_z in the experiments are monomers, dimers or oligomers, and whether they are homomers or heteromers is presently unknown. Receptor heteromerization has been observed to result in the creation of receptor complexes with novel signalling properties, where ligand binding affinities, signalling efficacies, G protein coupling preferences, as well as receptor desensitization and trafficking characteristics can change (George et al., 2000; Rios et al., 2001). For instance, when the μ and δ opioid receptors are expressed alone, the agonist detected high affinity receptor binding states, together with agonist induced inhibition of adenylate cyclase, are sensitive to pertussis toxin, indicating that the receptors are coupled to $G_{i/o}$ proteins. However, co-expression of the two receptors caused both the high affinity receptor binding state and adenylate cyclase inhibition to become pertussis toxin resistant, suggesting that the receptors are now coupled to another G protein (George et al., 2000). Based on present knowledge, this G protein preferred by the μ-δ heteromer is likely to be G_z since G_z is the only currently known pertussis toxin insensitive G protein that is capable of coupling to plasma membrane bound receptors to produce inhibition of adenylate cyclase.

1.4.3 Other types of receptors

Conventional activators of G protein signalling are receptor proteins with seven transmembrane segments (Bockaert et al., 2002). Evidence accumulated over the past decade, however, has expanded the list to include receptors with a single transmembrane spanning domain. Examples include the insulin receptor, which stimulates $G_{q/11}$ (Imamura et al., 1999), the insulin-like growth factor 1 receptor, which activates G_i (Dalle et al., 2001) and the NKR-P1 receptor, which has been found to couple to G_{i3}, G_s, $G_{q/11}$, and G_z (Al Aoukaty et al., 1997). The demonstration that the NKR-P1 receptor can couple to four G proteins was based on evidences obtained using IANK cell membranes. Stimulation of the NKR-P1 receptor in IANK cells significantly enhanced GTPγS binding to $G\alpha_{i3}$, $G\alpha_s$, $G\alpha_{q/11}$ and $G\alpha_z$, but not to $G\alpha_o$, $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{12}$ and $G\alpha_{13}$ (Al Aoukaty et al., 1997). Immunoprecipitation of the receptor protein

with antibodies against various G protein α subunits also demonstrated that $G\alpha_{i3}$, $G\alpha_s$, $G\alpha_{q/11}$ and $G\alpha_z$ can associate with the NKR-P1 receptor (Al Aoukaty et al., 1997). However, as all of the above evidence was based on data using broken membrane preparations, it is not known whether the coupling between the NKR-P1 receptor and the four G proteins that has been observed would also occur in intact cells. The presence of protein scaffolds and/or cytoskeletal elements in intact cells may limit the range of G proteins that a receptor can gain access to, and thus increase signalling specificity (Dumont et al., 2002; Oh and Schnitzer, 2001).

IANK cells are involved in the lysis of allogeneic and tumor target cells (Maghazachi et al., 1996). To lyse cells, IANK cells need to identify their targets and such target identification is critically dependent on cell surface receptors. The NKR-P1 receptor is an example of such a receptor, and if G_z is really a signal transducer for the receptor, one may expect target cell recognition and lysis to be affected when G_z is prevented from performing its normal function. When antibodies against $G\alpha_z$ were incorporated into permeabilized natural killer cells, there was a significant reduction in the lysis of allogeneic and tumor target cells (Maghazachi et al., 1996), consistent with G_z playing a role. In contrast, incorporation of antibodies against $G\alpha_s$ and $G\alpha_q$ did not affect target cell lysis (Maghazachi et al., 1996), suggesting that they either do not couple to the NKR-P1 receptor *in vivo* or they may mediate other functions of the NKR-P1 receptor.

1.4.4 Receptor independent G protein activators

In addition to receptors, there are many other accessory proteins and molecules that can influence the guanine nucleotide exchange reaction on the G protein α subunit. These include volatile anesthetics (e.g. halothane), which suppress GTP binding to $G\alpha_i$ (Pentyala et al., 1999); type V adenylate cyclase, which facilitate receptor mediated activation of G_s and G_i (Scholich et al., 1999; Wittpoth et al., 2000); tubulin, which enhances the exchange of GTP for GDP on $G\alpha_s$ and $G\alpha_{i1}$ (Popova et al., 1994); the neuronal growth associated protein (GAP-43) and β amyloid precursor protein, which can activate G_o independent of the receptor (Okamoto et al., 1995; Strittmatter et al., 1991); a family of proteins known as Activators of G protein Signalling (AGS), which are capable of activating G proteins based on various receptor independent mechanisms

(Cismowski et al., 2001); and the free radical OH; which directly activates G_i and G_o (Nishida et al., 2000; Nishida et al., 2002).

The capacity of many of these agents to modify the guanine nucleotide exchange activity of $G\alpha_z$ has not been examined. However, in the case of the OH free radical, the activation mechanism is known to consist of two steps. In the first step, OH modifies a cysteine residue at position 287 of $G\alpha_{i/o}$, leading to dissociation of $G\alpha$ from $G_{\beta\gamma}$. The second step entails modification of another cysteine at position 326 of $G\alpha_{i/o}$. This causes dissociation of GDP and binding of GTP to the α subunit (Nishida et al., 2002). Both of these cysteine residues are conserved in $G\alpha_z$. This may therefore represent an additional mechanism through which activation of G_z can occur.

1.5 Effectors of $G\alpha_z$

1.5.1 Adenylate cyclase

Since $G\alpha_z$ is a member of the $G\alpha_i$ family, it may be expected to share some of the same molecular effectors as $G\alpha_i$. $G\alpha_i$ was originally discovered and named based on its ability to inhibit the enzyme adenylate cyclase (Bokoch et al., 1984; Wong et al., 1991; Wong et al., 1992). Therefore, it was not surprising that adenylate cyclase became the first downstream effector identified for $G\alpha_z$. The expression of $G\alpha_z$ in human embryonic kidney 293 cells (which do not contain $G\alpha_z$) was found to cause agonist induced inhibition of adenylate cyclase activity, which was normally pertussis toxin sensitive, to become resistant to the toxin (Wong et al., 1992). To verify that these results were due to the α subunit of G_z, and not to the $\beta\gamma$ subunits, both of which would be activated by the agonist, a mutationally activated form of $G\alpha_z$ ($G\alpha_z$ Q205L), where a conserved glutamine residue in the GTPase domain was replaced with leucine, was employed. Expression of this $G\alpha_z$ mutant was found to produce dose dependent inhibition of adenylate cyclase activity, confirming $G\alpha_z$'s role in the suppression of cAMP accumulation (Wong et al., 1992). To date, there are ten distinct isoforms of adenylate cyclase identified in mammals (adenylate cyclase types I to IX and soluble adenylate cyclase) (Hanoune and Defer, 2001). $G\alpha_z$ has been found to be capable of

only inhibiting adenylate cyclases types I, V and VI (Ho et al., 2000; Kozasa and Gilman, 1995), all of which are also inhibited by the $G\alpha_i$s (Hanoune and Defer, 2001).

1.5.2 Rap1GAP

The GAP of the small monomeric G protein, Rap1, has been shown to be another downstream effector of $G\alpha_z$. Rap1GAP binds $G\alpha_z$ only in its active GTP bound form, and not in the inactive GDP bound form (Meng et al., 1999). The binding of Rap1GAP to activated $G\alpha_z$ has no effect on the sluggish GTP hydrolysis rate of $G\alpha_z$, but instead, attenuates the G_z GAP activity of RGSZ1 and RGS10 (Meng et al., 1999). The result is therefore a potential lengthening of the G_z signal. However, Rap1GAP also reduce the capacity of activated $G\alpha_z$ to inhibit type V adenylate cyclase (Meng et al., 1999). Currently, it is not known whether the ability of $G\alpha_z$ to inhibit the type I and VI isoforms of adenylate cyclase will be similarly attenuated. If the attenuation applies to all forms of adenylate cyclases that $G\alpha_z$ interacts with, it may cancel out the effect of prolonged $G\alpha_z$ signalling on adenylate cyclase activity brought about by the inhibition of G_z GAP by Rap1GAP. However, signalling to other $G\alpha_z$ effectors and $G_{\beta\gamma}$ effectors may still be prolonged.

The binding of $G\alpha_z$ to Rap1GAP does not interfere with Rap1GAP's interaction with Rap1, as Rap1GAP binds the two proteins on different parts of the molecule (Meng et al., 1999). The precise amino acid residues that interact with $G\alpha_z$ has not been mapped, but it has been found to occur within the first seventy four amino acids of Rap1GAP (Meng et al., 1999). This amino terminal region of Rap1GAP has recently been found to contain a G protein regulatory (or Goloco) motif, which binds selectively to members from the $G\alpha_i$ family (Natochin et al., 2001; Siderovski et al., 1999). It is therefore likely that $G\alpha_z$ interacts with Rap1GAP through this binding motif.

Although Rap1 has a ubiquitous tissue distribution, it is found in particularly high abundance in blood platelets, neutrophils and brain (Bos, 1998). Nervous tissues and blood platelets are the two principal sources of $G\alpha_z$ (Casey et al., 1990), and it is possible that the interaction between $G\alpha_z$ and Rap1 in these tissues may be functionally significant. In blood platelets, stimulation of $G\alpha_z$ has been found to cause increased

activation of Rap1, which may play a role in platelet activation (Woulfe et al., 2002). The mechanism by which stimulation of $G\alpha_z$ leads to Rap1 activation in platelets is still unknown. Since activated $G\alpha_z$ can bind to Rap1GAP, it has been suggested that the increased activation of Rap1 may be due to an inhibition of Rap1GAP activity by activated $G\alpha_z$ (Woulfe et al., 2002). Preliminary data, however, suggests that the binding of $G\alpha_z$ to Rap1GAP does not modify the GAP activity of Rap1GAP towards Rap1 (Meng et al., 1999). An alternative mechanism is the recruitment of Rap1GAP by $G\alpha_z$ causes Rap1GAP to be relocated to particular subcellular compartments, which decreases its opportunity of interacting with activated Rap1 (Meng et al., 1999). These and other possibilities will need to be explored in future research.

In nervous tissues, activation of Rap1 results in the stimulation of extracellular signal regulated kinase (ERK) (also known as mitogen activated protein kinase or MAP kinase), which is involved in the regulation of synaptic plasticity and neuronal survival (Grewal et al., 1999). The interaction between $G\alpha_z$ and Rap1 in nervous tissues has not been examined. However, the lack of $G\alpha_z$ in sympathetic neurons attenuated nerve growth factor (NGF) dependent neuronal survival in the presence of pertussis toxin (Powell et al., 2002). NGF has been shown to activate Rap1 (Yao et al., 1998), and this results in sustained activation of ERK (York et al., 1998), which likely contribute to the survival of sympathetic neurons (Xue et al., 2000). As in blood platelets, stimulation of $G\alpha_z$ may result in greater Rap1 activation (Woulfe et al., 2002), which in turn contributes to improved sympathetic neuron survival in the presence of pertussis toxin (Powell et al., 2002). When pertussis toxin is absent, stimulation of $G\alpha_i$, which is also capable of activating Rap1 (Woulfe et al., 2002) and cause stimulation of the ERK signalling cascade (Mochizuki et al., 1999), may compensate for the loss of $G\alpha_z$. Interestingly, NGF has been found to cause persistent activation of Rap1 in endosomes, which is prevented by disruption of Golgi and endosomal compartments with brefeldin A (Wu et al., 2001). These NGF containing endosomes have been proposed to serve as signalling organelles for transportation of the NGF survival signal back to the cell body of neurons with long axons (Grimes et al., 1997; Sandow et al., 2000). Both $G\alpha_i$ and $G\alpha_z$ have been found to be retrogradely transported by neurons (Hendry et al., 1995a). However, their co-localization with Rap1 and NGF has never been investigated. It is possible that they may bind to Rap1GAP and Rap1 in the same signalling organelle, to modulate the activation state of Rap1.

1.5.3 G protein Regulated Inducer of Neurite outgrowth (GRIN)

G protein Regulated Inducer of Neurite outgrowth 1 (GRIN1) and GRIN2 are two downstream effectors of G protein α subunits based on their preferential interaction with the activated forms of $G\alpha_z$ and $G\alpha_o$ (Chen et al., 1999). Besides $G\alpha_z$ and $G\alpha_o$, GRIN1 has also been found to bind to $G\alpha_{i1}$ and $G\alpha_{12}$, but not $G\alpha_s$ or $G\alpha_q$ (Chen et al., 1999). When GRIN1 or GRIN2, together with a mutationally activated form of $G\alpha_o$ ($G\alpha_o$ Q205L) were co-transfected into embryonic kidney MA104 and neuroblastoma Neuro2a cells, both proteins were found to be capable of inducing the formation of neurite-like processes in these cells (Chen et al., 1999). However, whether the interaction of GRIN1 or GRIN2 with activated $G\alpha_z$ would also trigger neurite extension, has not been investigated. Based on subcellular distribution analysis of membranes from embryonic mouse brains, GRIN1 and $G\alpha_o$ were found to co-localize and were particularly enriched in growth cone membranes while $G\alpha_z$ was distributed throughout all membrane fractions (Chen et al., 1999). A possible role of activated $G\alpha_z$ may therefore be to sequester GRIN1, preventing its interaction with activated $G\alpha_o$ and accordingly, regulate neurite growth and the morphology of the cell.

1.5.4 Eyes Absent transcription cofactor Eya2

Eya2, the mammalian homologue of the drosophila eyes absent (Eya) protein, is another downstream effector of $G\alpha_z$. Eya2 was found to interact specifically with the activated forms of $G\alpha_z$ and $G\alpha_{i2}$, but not with activated $G\alpha_s$ or the GDP bound forms of the α subunits, suggesting it could potentially serve as another effector for members of the $G\alpha_i$ family (Fan et al., 2000). The binding of $G\alpha_z$ or $G\alpha_{i2}$ to Eya2 prevents Eya2 from interacting with its other partner, a member from the Six protein family (Fan et al., 2000). Normally, the docking of Eya2 to a Six protein would result in the complex being translocated from the cytosol into the nucleus, where it induces gene transcription (Ohto et al., 1999). The presence of activated $G\alpha_z$ and $G\alpha_{i2}$, however, suppresses this translocation and causes Eya2 to remain in the cytoplasm (Fan et al., 2000).

In drosophila, Eya is essential for development of the compound eye. A deficiency in Eya causes progenitor cells in the eye disc anterior to the morphogenetic furrow, to undergo apotosis, instead of differentiation (Bonini et al., 1993). Since eye development is evolutionarily conserved between flies and humans, it is likely that the mammalian homologues of Eya will also have some role in vertebrate eye development (Fee et al., 2002). Consistent with this, Eya2 has been found to be extensively expressed in the mouse central nervous system, including all crancial placodes, which give rise to the eyes, the nose and various sensory ganglia during organogenesis (Xu et al., 1997). The level of Eya2 expression is also maintained in the placode derived structures until at least E16.5 (Xu et al., 1997). The interactions of $G\alpha_{i2}$ and $G\alpha_z$ with Eya2 during development have not been examined. However, it is interesting to note that the expression level of $G\alpha_z$ is very low in the brain and various sensory ganglia at E15, and the level increases from then on, to peak at around two to three weeks after birth (Kelleher et al., 1998). The very low expression level of $G\alpha_z$ in the central nervous system early in mouse development may be important to prevent suppression of Eya2 mediated gene transcription, so that organogenesis can proceed. Later in development, it is possible that receptor mediated activation of $G\alpha_z$ (and $G\alpha_{i2}$) induced by molecules secreted by other cells into the extracellular matrix may play a role in regulating the transcription activity of Eya2.

1.6 Examination of G_z signalling through the $G\alpha_z$ knockout mouse

The review of the literature presented above suggests that $G\alpha_z$ is a protein with unique biochemical properties. Therefore, the signalling pathways activated by G_z may serve a special role in those tissues in which $G\alpha_z$ is expressed. However, most of the research on G_z has been performed *in vitro*. The investigation of its functions *in vivo* has been hampered by the lack of pharmacological tools, and its intracellular localization (which means cells may need to be destroyed to allow antibodies to gain access to the protein).

Gene targeting is a very powerful molecular biological tool that has gradually been developed over the last twenty-five years (Capecchi, 2001). The technique allows a very 'clean' and precise ablation of a protein, which is otherwise difficult to achieve by the use of other methods (antibodies, antisense oligonucleotides). It also allows the impact of mutagenizing a gene on a whole organism to be studied, and therefore, enables the functions of the protein to be examined *in vivo*.

In an attempt to discover the physiological importance of signal transduction pathways mediated by G_z, my laboratory has adopted this technique to 'knockout' $G\alpha_z$ in mice. The inactivation of $G\alpha_z$ through disruption of its coding sequence, would result in a null protein, and prevent the assembly of G_z in cells. This work was started during my honours year in 1993, when I successfully cloned the mouse $G\alpha_z$ cDNA (Leck, 1993). When I returned in 1999, the $G\alpha_z$ knockout mouse had already been generated. Dr. Kim Powell (nee Kelleher), for her Ph.D. studies, was examing the phenomenon of opioid tolerance in these mice. At that time, she has already successfully demonstrated that the $G\alpha_z$ knockout mouse develop greater morphine tolerance in the hotplate test, but not in the tail flick test. I suggested that we examine the responses of these mice to cold pain since there is evidence that morphine may affect spinal nociceptive neurons sensitive to 'intense cold' differently from other nociceptive stimuli (Casey, 1996; Craig and Hunsley, 1991). We did not find any difference in the responses of the $G\alpha_z$ knockout mouse to 'hot' or 'cold' pain, strengthening our hypothesis that the greater tolerance to morphine that developed in the $G\alpha_z$ knockout mouse is mediated supraspinally. This work has subsequently been published (Hendry et al., 2000). In this thesis, I present work, which I have performed from then on, whereby I examine the mechanisms underlying the increased morphine tolerance in the $G\alpha_z$ knockout mouse. The results from biochemical assays using broken membrane preparations to study G protein activation by morphine and other opioid agonists have largely been unsuccessful, as I could not find any consistent difference between tolerant wildtype and $G\alpha_z$ knockout brain membranes. This is despite performing the assay under a variety of different ionic concentrations of magnesium and sodium, which may affect the activation kinetics of different G proteins (Casey et al., 1990). Nonetheless, I have ruled out pharmacokinetic and behavioural mechanims as explanation for the greater morphine tolerance in $G\alpha_z$ knockout mice (Chapter 3). I have also extended the research to examine other effects of morphine in these mice: including physical dependence (Chapter 3), locomotor activation (Chapter 4) and thermoregulation (Chapter 6). Furthermore, since the $G\alpha_z$ knockout mouse showed increased locomotor activity in response to cocaine (Yang et al., 2000) and there is evidence that G_z can couple to dopamine D2-like receptors *in vitro* (Obadiah et al., 1999; Wong et al., 1992), I have also examined the functions of dopamine D2-like receptors in these mice (Chapter 5).

Chapter 2

Materials and Methods

2.1 Animals

Mutant $G\alpha_z$ knockout mice were derived from heterozygous founders generated originally by gene targeting in the C57BL/6 mouse strain as described (Hendry et al., 2000). Briefly, a 1.8kb Hind III- Eco RI fragment containing the PGKneo^r cassette was blunt-end cloned into a Bsa B1 site located 160bp downstream of the translation start site in a 3.8kb genomic fragment of the $G\alpha_z$ gene. A 2.84 kb Hind III-EcoRI fragment of the PGK.TK cassette was added to the 3' end of the targeting vector. The linearised vector was electroporated into C57BL/6 embryonic stem cells and homologous recombinants were identified by positive-negative selection (Mansour et al., 1988) and a polymerase chain reaction (PCR) based strategy using nested primers, (Nitschke et al., 1993). Verification of the targeting was made by Southern blot analysis of genomic DNA from selected ES cells restricted with KpnI or Hind III-Eco R1 and the targeted clones shown to have only one site of integration of the neo^r gene. Chimæras were obtained by blastocyst injection and shown to transmit the targeted gene through the germline. Heterozygous mice were inter-crossed to obtain mice homozygous for the targeted $G\alpha_z$ gene.

Mice used for the experiments, although of pure C57BL/6 origin, were crossed for 8 generations into the C57BL/6 background to remove any random mutations that might have occurred in the parental C57BL/6 stem cells used for electroporation. The animals were housed 4-6 to a cage in a temperature-controlled room maintained at 23±2°C, with food and water available *ad libitum*. All experiments were performed on age and gender matched C57BL/6 mice, using protocols approved by the Animal Experimentation Ethics Committee of the Australian National University or the Macquarie University. Adult mice were between 3 and 8 months of age, while pre-adolescent mice were between 24 and 27 days old. Male mice were used in all experiments, except in Chapter 3, where gender differences in the analgesic response to morphine was examined, and in Chapter 5 for the experiment on measurement of ACTH released by various drugs, where equal numbers of wildtype and $G\alpha_z$ knockout females

were included to make up for insufficient mouse numbers. Where possible, mice of different genotypes, but comparable age were evaluated in parallel in the experiments. When this was not possible due to shortage of resources, wildtype and $G\alpha_z$ knockout mice were evaluated alternately.

2.2 Drugs

Nor-binaltorphimine (Nor-BNI), U-50,488 hydrochloride (trans-(dl)-3,4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)cyclohexyl]-benzeneacetamide) and 75mg morphine base pellets were generous gifts from Kevin Gormley at the Research Triangle Park, NIDA, Bethesda, Maryland, USA Other drugs were purchased: *d*-amphetamine sulfate (May and Baker, UK), quinpirole (Sigma, St. Louis, MO, USA), 8-hydroxy-dipropylamino-tetralin (8OH-DPAT) (Sigma, St. Louis, MO, USA), SKF 38393 (Tocris Cookson, Bristol, UK), corticotropin releasing hormone (CRH) (Sigma, St. Louis, MO, USA), morphine tartrate (David Bull Laboratories, Australia), morphine hydrochloride (Macfarlan Smith, U.K.), naloxone hydrochloride (ICN Biomedicals, Aurora, OH, USA) and haloperidol (Sigma, St. Louis, MO, USA).

All drugs were dissolved in physiological saline and administered in a volume of 10 µl/gram mouse weight subcutaneously unless otherwise stated. Morphine tartrate (David Bull Laboratories, Australia) was administered in all antinociceptive assays and morphine hydrochloride (Macfarlan Smith, U.K.) was used to induce tolerance during chronic morphine treatment. All morphine concentrations reported were the concentrations of the respective morphine salts. Haloperidol was dissolved in a drop of acetic acid and diluted with saline.

2.3 Chemicals

All chemicals were obtained from Sigma (St. Louis, MO, USA) or ICN Biomedicals (Aurora, OH, USA) unless otherwise stated.

2.4 Antibodies

The antibodies against $G\alpha_z$ were made by Professor Ian Hendry and Dr. Michael Crouch at the John Curtin School of Medical Research (Australian National University, Canberra, Australia). Antibodies against $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_o$, $G\alpha_s$, $G\alpha_q$, $G\alpha_{i\ (common)}$, and $G\beta_{(common)}$ were generous gifts from Dr Michael Crouch. The $G\beta_{(common)}$ antibody recognized a common peptide sequence found in $G\beta_1$, $G\beta_2$ and $G\beta_3$.

2.5 Western Blotting

Mouse brain homogenates were prepared at a concentration of 20% (w/v) in SDS (sodium dodecyl sulphate) sample buffer (10% glycerol, 2.5% dithiothreitol, 3% SDS, bromophenol blue, 0.5M Tris-HCl, pH 6.8) and spun in a refrigerated centrifuge at 10,000g for 20 minutes. Forty µl of supernatant containing 0.5 mg protein were loaded onto a 9% SDS-polyacrylamide gel. After gel electrophoresis, the resolved proteins were electro-transferred onto a nitrocellulose support. The nitrocellulose was blocked with 5% skim milk at room temperature for 1 hour, and then incubated with the appropriate primary antibody at 5°C overnight. The blot was rinsed four times with phosphate buffered saline containing 0.1% (v/v) Tween20 (PBST) for 15 minutes each, then incubated with horseradish peroxidase conjugated secondary antibody (Amersham Pharmacia, United Kingdom) at room temperature for 1 hour. This was followed by extensive washing with PBST for four hours, with 4 changes. The bands were detected using the Enhanced Chemiluminescence kit (Amersham Pharmacia, United Kingdom) and visualized on a Phosphoimager (Fuji, Japan). Analysis of the amount of protein in each band was performed using the ScienceLab Image Gauge Software (Fuji, Japan).

2.6 The Hotplate Assay

The hotplate apparatus comprised a cylinder (13.2 cm high and 15.2 cm in diameter) with a metallic floor plate maintained at a temperature of 52 ± 0.5°C. Animals were picked up by their tails and placed gently onto the cylinder floor. An observer blind to the genotype of the mouse recorded the time taken for each mouse to respond by either licking of its hindpaw or jumping from the hotplate. To minimize tissue damage, a maximum response latency of 150 seconds was recorded and the mouse removed from the hotplate if it failed to respond within this time period. The antinociceptive response

was expressed as a percentage of the maximum possible effect (%MPE), calculated for each mouse based on the following formula: [(post-drug latency – baseline latency)/ (cut off latency – baseline latency) * 100].

2.7 Morphine Treatment and Testing

Mice were tested on the hotplate during the mornings of the first, fourth and seventh day of morphine treatment. Three pre-drug trials were conducted immediately before morphine administration. The first reading was discarded, while the later two (where the animals were injected with physiological saline) were averaged to obtain the baseline response latency. Following this, mice were injected intra-peritoneally with successive doses of morphine tartrate and tested every twenty minutes to generate a cumulative dose response curve (Sora et al., 1997). The highest cumulative dose of morphine tartrate administered were 56mg/kg on Day 1, 220mg/kg on Day 4 and 370mg/kg on Day 7. To induce tolerance, these doses were supplemented with subcutaneous morphine hydrochloride injections on the evenings of day 1 (50mg/kg), day 4 (200mg/kg) and day 7 (300mg/kg), as well as on the mornings and evenings of day 2 (100mg/kg), day 3 (150mg/kg), day 5 (250mg/kg) and day 6 (300mg/kg). In summary, the following total cumulative dose of morphine were administered to mice over a seven day period (in mg/kg: Day 1:106, Day 2:200, Day 3: 300, Day 4: 420, Day 5: 500, Day 6: 600, Day 7: 370 (morning only)).

For the induction of morphine tolerance in mice that were not used for the hotplate assay, a similar protocol as that described above was followed, except that the cumulative morphine dosing the mice were supposed to receive on the mornings of days 1, 4 and 7 was substituted with a large bolus dose of morphine hydrochloride (day 1: 50mg/kg, day 4: 200mg/kg, day 7: 300mg/kg). Mice used for the naloxone precipitated withdrawal experiments were administered a further 300mg/kg of morphine hydrochloride on the evening of day 7 before being assessed for physical dependence on day 8.

The pellet implant method was used to induce morphine tolerance in some mice. With this protocol, each mouse was implanted subcutaneously with one 75mg morphine base pellet under light ether (APS Ltd, NSW, Australia) anaesthesia. They were then left to recover under a hot lamp for 30 minutes.

2.8 Morphine lethality

Tolerant wildtype and $G\alpha_z$ knockout mice chronically treated with morphine for 7 days were administered subcutaneously with a high dose of morphine hydrochloride (700 to 900mg/kg) and the percentage of mice that died within 6 hours from the injection was recorded.

2.9 Naloxone precipitated withdrawal

Mice chronically treated with morphine or saline for 7 days were administered 50mg/kg morphine HCl or saline on the morning of day 8. Two hours later, the mice was injected intraperitoneally with 4mg/kg naloxone HCl. Immediately following the injection, each mouse was placed in a large transparent plexiglas cage (48cm x 24cm) divided by 3 lines lengthwise and one line breadthwise into 8 squares. Two observers blind to the genotype and drug treatment condition of the mice counted the number of bouts of jumping, wet dog shakes and line crossings over a 15-minute time period. Body weights were determined before the administration of naloxone and 60 minutes later.

2.10 Measurement of cyclic AMP levels in brain tissues

Male wildtype and $G\alpha_z$ knockout mice were chronically treated with morphine or saline for 7 days. On day 8, they were given another dose of 50mg/kg morphine HCl or saline. Two hours later, 4mg/kg naloxone HCl was administered. The mice were sacrificed after 20 minutes by rapid decapitation and the head was immediately irradiated in a 1kW microwave for 3 seconds to inactivate phosphodiesterases (Lenox et al., 1977). The striatum and periaqueductal gray (PAG) were dissected on ice, and cyclic AMP was extracted with ethanol. Tissue cyclic AMP levels were measured using a radioimmunoassay kit (Amersham Pharmacia, United Kingdom). Tissue protein was estimated using a protein assay kit (Bio-rad, Hercules, CA, USA).

2.11 Morphine pharmacokinetic studies

Tolerant mice treated with morphine for 6 days following the protocol described above were administered 50mg/kg of morphine on the morning of day 7. About two hours after the last morphine injection, mice were killed by an over-dose of ether and blood was drawn from the jugular veins. The blood was allowed to clot at 37°C for 1 hour, then kept in a refrigerator for 3 hours for the clot to contract. Subsequently, the supernatant was transferred to a new polypropylene tube, centrifuged at 246g at 4°C for 4 minutes, and the clear serum carefully pippetted into a new tube. Serum from saline injected animals was processed in parallel to serve as controls. All sera were stored at –20°C until sent by overnight courier on dry ice to Professor Maree Smith's laboratory at the School of Pharmacy, University of Queensland for further analysis.

2.12 Quantification of morphine and M3G in mouse serum

Serum concentrations of morphine and morphine-3-glucuronide (M3G) were quantified using high-performance-liquid-chromatography with mass spectrometric detection (HPLC-MS/MS) at Professor Maree Smith's laboratory (Wright et al., 2000). Aliquots of serum (50 μl) were added to 10ml polypropylene tubes, followed by 100μl of internal standard (hydromorphone, 2 ng/μl). Samples were deproteinated by the addition of 150 μl of acidified (1% formic acid) acetonitrile, which was followed by vortex mixing for 10 seconds. After centrifugation, the supernatant was decanted into clean Eppendorf™ tubes and the fluid was removed using a Savant centrifugal vacuum dryer. The dried residues were then re-constituted with 50μl aliquots of deionised water (18.2 MΩ), followed by vortex mixing for 30 seconds. Sample aliquots (20μl) were then injected onto the HPLC-MS/MS and the compounds of interest were separated from endogenous components of plasma using a Zorbax SB-C_{18}, 5 μm 2.1 x 50 mm stainless steel column and an isocratic mobile phase comprising methanol: 0.1% formic acid (9:91) at a flow rate of 0.15 ml/min. The HPLC-MS/MS system comprised a Shimadzu VP HPLC system, and a Perkin-Elmer API 300 mass spectrometer. This assay was highly specific as each drug of interest was identified and quantified using selected ion monitoring according to the mass ratio of the parent ion to the daughter ion as follows, viz. morphine 286.2/201.1, M3G 462.5/286.2, hydromorphone 286.4/184.6. The recoveries for morphine and M3G from serum were 74% and 58% respectively (Wright et al.,

2000) and the lower limits of quantification for morphine and M3G were 156 and 313pg injected on-column respectively.

Standard curves comprising six to seven concentrations of morphine and M3G were processed in random order with each batch of mouse serum samples. Regression analysis was used to produce standard curves, which were accepted if the correlation coefficients were ≥ 0.995. Intra-day and inter-day coefficients of variation (%CVs) for control serum samples containing added morphine (3.9 ng/ml) and M3G (7.8 ng/ml) in concentrations that were identical to those used for the lower limit of quantification were <15% (Wright et al., 2000).

2.13 Measurement of body temperature

Mice were housed individually in separate cages for about one hour prior to the start of each experiment, which took place between 1330 and 1530hrs in the afternoon. Body temperature was measured with a rectal thermister probe (Physitemp Instruments, Clifton, New Jersey, USA). The probe was inserted into the rectum to a depth of about 3cm, which provided a reliable measure of the animal's core body temperature. To habituate the mice to the testing procedure, the body temperature of the mouse was measured every 15 minutes for one hour prior to drug administration.

2.14 Measurement of plasma corticosterone levels

Mice were housed individually overnight in separate cages. The collection of blood samples via retro-orbital bleeding always occurred between 0900hrs and 1000hrs the next morning, and was performed within forty seconds from initial disturbance of the cage. Mice were bled either without any injection (basal measurement), 15 minutes after subcutaneous injection with saline vehicle or at the indicated time intervals after 50mg/kg of subcutaneous morphine hydrochloride. About 100μl of blood was collected into ice-cold tubes containing 50 Kallikrein inhibitor unit of aprotinin and 2.5μl of 6% ethylenediaminetetraacetic acid. The blood was centrifuged at 246g for 20 minutes at 4°C, and the plasma stored in a -70°C freezer until ready for hormonal analysis. The level of corticosterone in blood plasma was determined using a radioimmunoassay kit obtained from ICN Biomedicals (Aurora, OH). In some experiments, the corticosterone

synthesis inhibitor, metyrapone was administered subcutaneously two hours prior to morphine treatment.

2.15 Measurement of ACTH release induced by different drugs

Mice were housed individually overnight in separate cages. The collection of blood samples via retro-orbital bleeding took place between 0900hrs and 1000hrs, and was performed within forty seconds from initial disturbance of the cage. Mice were bled either without any injection (basal measurement), or 15 minutes after subcutaneous injection with saline vehicle, 0.5mg/kg 8-hydroxy-dipropylamino-tetralin (8OH-DPAT), 1mg/kg quinpirole or 30 minutes after intraperitoneal injection with saline vehicle or 10μg/kg CRH. About 100μl of blood was collected into ice-cold tubes containing 50 Kallikrein inhibitor units of aprotinin and 2.5μl of 6% ethylenediaminetetraacetic acid. The blood was centrifuged at 246g for 20 minutes at 4°C, and the plasma stored in a -70°C freezer until ready for hormonal analysis. The level of ACTH in blood plasma was determined using a radioimmunoassay kit obtained from ICN Biomedicals (Aurora, OH, USA). As the level of ACTH did not differ when blood samples were collected either 15 or 30 minutes after saline injection, the data were combined during data analysis to obtain a single mean control value for mice of each genotype.

2.16 Automated measurement of locomotor activity

Mice were accustomed to the testing room for two hours prior to the start of each experiment. Locomotor activity measurement always commenced between 1330 and 1530hrs in the afternoon. After the injection of a drug or saline vehicle, each mouse was placed immediately into a cage (29cm x 18cm) fitted with 2 pairs of infra-red photocells positioned 1.5 cm above the floor and spaced 10cm apart. The cage was made to look identical to the home cage, with sawdust, food and water. The set up allows the locomotor activity of eight mice (four wildtype and four $G\alpha_z$ knockouts) to be evaluated simultaneously in eight cages separated visually from one another.

2.17 Analysis of locomotor activity profile

In Chapter 4, the morphine stimulated locomotor activity profile of each mouse was analysed by measuring three parameters: (i) the total number of beam breaks during the

first hour after morphine administration, (ii) peak activity and (iii) duration of response. Peak activity is operationally defined as the maximum number of beam breaks in any 15-minute time bin. The duration of the morphine response was measured by the time it took for the locomotor activity of a morphine treated mouse to return to within two standard errors from the activity average of saline treated mice.

2.18 Constant Potential Amperometry

Each mouse was anaesthetized with an intraperitoneal injection of 1.5g/kg urethane. The mouse's head was shaved and carefully placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA) containing a mouse head-holder adaptor (Stoelting, Kiel, WI, USA). The body temperature of the mouse was maintained at 40 ± 0.5°C with a temperature-regulated heating pad (TC-831; CWE Inc., New York, NY). Three holes were drilled through the animal's skull: one for the reference/auxiliary electrode, another for the stimulating electrode and a third relatively larger hole for both the recording electrode and an adjacent 31g stainless-steel guide cannula. The tip of a concentric bipolar stimulating electrode (SNE-100; Rhodes Medical Co., Woodland Hills, CA, USA) was positioned in the medial forebrain bundle (MFB) 2.1mm posterior to the bregma and 1mm lateral to the medial line (Franklin and Paxinos, 1997). The depth of the electrode was about 4.3mm from the dura, and was adjusted for every experiment so that the release of dopamine was close to maximum. The recording electrode, which utilized a carbon fibre (10μm thick and 250μm long, Thornel Type P, Union Carbide, USA) as the active recording surface, was implanted into the core region of the ipsilateral nucleus accumbens. The coordinates were 1.5mm anterior to the bregma and 1mm lateral to the medial line. Again, the depth, which was between 3.3 to 3.5mm from the dura, was adjusted for each experiment to maximize dopamine release. For drug infusion, a guide cannula was implanted as close to the recording electrode as possible (0.2 to 0.3 mm), with its end positioned 1mm above the tip of the recording electrode, so that when the infusion cannula (100μm o.d., Polymicro Tech. Inc., AZ, USA) was inserted, its tip was approximately in line with the middle of the recording electrode's carbon fiber. The tip of the reference/auxiliary electrode combination was positioned through one of the contralateral skull holes and placed in contact with cortical tissue.

The recording electrode was left to equilibrate in the tissue for approximately one hour. After the recording electrode showed stable stimulation-evoked dopamine responses, a baseline response profile for each mouse was acquired by applying a train of monophasic 0.5msec duration pulses (15 pulses at 50Hz, 500μA) once every thirty seconds for 2.5 minutes. Six stimulation-evoked responses were recorded automatically by a Macintosh computer running the 'Chart' program (ADInstruments, Castle Hill, NSW, Australia) and were subsequently averaged to obtain a mean baseline response. Thereafter, 100ng of quinpirole in a volume of 1μl was infused slowly into the nucleus accumbens through the injection cannula via a 10μl microsyringe over a time period of 4 minutes. At 15 minute time intervals from the start of infusion, a mean dopamine release profile was obtained by averaging the responses from six stimulating pulse trains, following exactly the same protocol as was used for obtaining the baseline response. Recordings continued for at least 90 minutes after the start of quinpirole infusion.

Upon the completion of each experiment, an iron deposit was made in the MFB stimulation site by passing direct current (100μA for 10sec) through the stimulating electrode. The mouse was then euthanized with an overdose of urethane (3g/kg). The brain was removed, immersed overnight in 10% buffered formalin containing 0.1% potassium ferricyanide (Lancaster Inc., Eastgate, UK), and then stored in a solution containing 30% sucrose and 10% formalin until sectioning. After fixation, 40μm coronal sections were cut on a freezing microtome. A Prussian blue spot, which is produced from a redox reaction of ferricyanide, marks the stimulation site. The placements of the electrochemical recording electrode, stimulating electrode and microinfusion cannulae were determined under a light microscope and recorded on representative coronal diagrams (Franklin and Paxinos, 1997).

2.19 Data Analysis.

All data analysis were performed with the Statistical Package for the Social Sciences (SPSS) software (SPSS Inc., Chicago, USA). For parametric data containing one factor with only two levels, data analysis was performed using the student's t test. For all other parametric data, the Analysis of Variance (ANOVA) procedure was used. To analyse gender differences in morphine analgesia and tolerance between mice of different genotypes (Chapter 3), repeated measures ANOVA was performed employing

morphine dose as a within subject factor and genotype as a between subject factor. In analysing the data of mice implanted with morphine pellets (Figure 3.4), time after morphine pellet implant was a within subject factor and genotype was a between subject factor. ANOVA of the data presented in Chapters 4, 5 and 6 was accomplished using time as a within-subject factor, drug dose and genotype as between-subject factors. All possible interactions between the factors were examined. However, in most cases, only interactions that were significant at $p<.05$ or better were reported. During the performance of repeated measures ANOVA, the Huynh-Feldt correction was applied when the sphericity assumption was violated. All post-hoc multiple comparisons were performed with Fisher's least significant difference procedure (Winer, 1970). For the non-parametric morphine withdrawal data presented in Chapter 3, the Kruskal-Wallis one-way ANOVA test was used. The plotted values in all figures are the means ± SEM.

Chapter 3

Morphine tolerance and physical dependence in the $G\alpha_z$ knockout mouse

3.1 Introduction

Morphine is a potent pain reliever. It is commonly used for alleviating post-surgical pain and is recommended by the World Health Organization for treating moderate to severe pain associated with advanced cancer (Collett, 1998; Zech et al., 1995). However, prolonged use of morphine often necessitates dose escalation even in the absence of disease progression, to achieve the same degree of analgesia, resulting in increased cost as well as risk of drug dependence. This rightward shift in the dose response curve is due to the development of drug tolerance, a complex clinical phenomenon that remains poorly understood.

Prolonged use of a drug can produce tolerance in a number of ways: Firstly, this can induce changes in its distribution and metabolism. This form of tolerance, also known as pharmacokinetic tolerance, is commonly caused by an increase in the rate of drug metabolism after repeated administrations. The consequent faster decline of its concentration in the blood and at its sites of action result in a smaller drug response. Additionally, if the metabolism of the drug produces metabolites with antagonistic properties, accumulation of these metabolites could lead to tolerance by counteracting the actions of the drug. In the case of morphine, one of its major metabolites, morphine-3-glucuronide (M3G), as well as a less abundant metabolite, normorphine-3-glucuronide (nor-M3G), have both been shown to attenuate morphine induced antinociception (Smith and Smith, 1998; Smith et al., 1990). The accumulation of both of these metabolites in the body could therefore oppose the pain relieving properties of morphine and potentially play a role in the development of tolerance (Smith and Smith, 1995).

A second form of tolerance is called pharmacodynamic tolerance. Morphine binds to opioid receptors, which in turn causes the activation of receptor bound G proteins, to inhibit adenylate cyclase, open potassium channels and produce other effects within the

cell (Standifer and Pasternak, 1997; Williams et al., 2001). Continuous exposure to morphine can promote adaptive changes in the receptors and cellular systems affected by the drug. These changes include short and long term receptor desensitisation, compensatory upregulation of adenylate cyclase activity, as well as adaptations in the neuronal circuitry involved with morphine effects (Williams et al., 2001). The contribution of one of these cellular mechanisms to morphine tolerance in a whole animal has been demonstrated in a mouse that lacks β-arrestin 2, a protein putatively involved in receptor desensitization. Compared to chronically morphine treated wildtype mice, mice deficient in β-arrestin 2 did not develop morphine tolerance and this was paralleled by an intact coupling of opioid receptors to G proteins, suggesting an absence of opioid receptor desensitization (Bohn et al., 2000).

Thirdly, in addition to pharmacological parameters, learning can also play a contributory role to the development of morphine tolerance (Ramsay and Woods, 1997). Associative tolerance develops through the repeated pairings of morphine administration with specific environmental cues (such as weighing before drug injection, testing for analgesia). Gradually, these cues acquire the properties of a conditioned stimulus and elicit a conditioned response that opposes the pharmacological effects of morphine. This diminution in morphine's effects appears as tolerance (Mitchell et al., 2000). Associative tolerance to the analgesic effects of morphine has been shown to require the stimulation of cholecystokinin B receptors in the amygdala, suggesting that the amygdala forms part of the circuitry that is responsible for developing this type of tolerance (Mitchell et al., 2000).

Besides causing tolerance, chronic morphine treatment also produces abstinence symptoms when the drug is abruptly withdrawn (physical dependence). These withdrawal symptoms include reduced locomotor activity, teeth chattering, diarrhea, wet dog shakes, body weight loss and jumping behaviour in rodents (Schulteis et al., 1994). The observation that the extent of morphine tolerance is related to the degree of physical dependence, has led to the hypothesis that opioid tolerance and physical dependence may share a common underlying mechanism (Way et al., 1969). Indeed, the frequent co-occurrence of drug tolerance and dependence is highlighted in a recent edition of the Diagnostic and Statistical Manual of Mental Disorders (American Psychiatric Association, 1994), where tolerance is listed as one of two main diagnostic criteria of physiological substance dependence. Currently, there is no good model to

explain physical dependence at the level of the whole organism. However, at the cellular level, the compensatory upregulation of adenylate cyclase activity in opioid sensitive neurons, as a result of prolonged morphine exposure, has been applied to explain both morphine tolerance and dependence (Collier, 1980; Finn and Whistler, 2001; Williams et al., 2001). According to this hypothesis, the decreased potency of morphine in the tolerant state, results from its lower efficacy to produce inhibition of cyclic AMP levels, due to a compensatory superactivation of adenylate cyclase activity within the cell. Withdrawal of morphine unmasks this latent upregulation of cyclic AMP levels, leading to a supra-basal rebound that reverses morphine's effects (Finn and Whistler, 2001). Superficially, some of the symptoms of morphine withdrawal, such as hyperalgesia (Laulin et al., 1999), reduced locomotor activity and diarrhea (Schulteis et al., 1994), appear opposed to the acute analgesic, locomotor activating (Sora et al., 2001a) and gastrointestinal anti-transit effects of morphine (Pol et al., 2001). Morphine dependence at the cellular level may play a role in the development of these symptoms.

Three types of opioid receptors have been cloned, and they have been designated using the Greek letters, μ, δ and κ (Kieffer, 1999) (Alexander et al. 2000). Although morphine can bind to all three receptors, it has the highest affinity for the μ receptor (Goldstein and Naidu, 1989; Raynor et al., 1994). Morphine analgesia and physical dependence are mediated through the μ receptor as these effects are abolished in the μ receptor knockout mouse (Matthes et al., 1996). The δ receptor, on the other hand, has been implicated in the development of morphine tolerance, as mice that lack the δ receptor did not develop tolerance to the analgesic effects of morphine to a significant degree (Zhu et al., 1999b). All opioid receptors are G protein coupled receptors. *In vitro*, the acute activation of all three receptors has been shown to inhibit adenylate cyclase through the stimulation of G_z or pertussis toxin sensitive $G_{i/o}$ (Ozawa et al., 1999; Tso et al., 2000; Tso and Wong, 2000b). Following chronic opioid treatment, there was a compensatory increase in adenylate cyclase activity that was sensitive to the effects of pertussis toxin (Ozawa et al., 1999; Tso et al., 2000; Tso and Wong, 2000a; Tso and Wong, 2000b; Tso and Wong, 2001). However, either G_{i1}, G_{i2}, G_{i3} or G_z alone was found to be insufficient to mediate the chronic opioid induced superactivation of adenylate cyclase activity. This suggests that the adenylate cyclase superactivation is either mediated by G_o or requires the simultaneous stimulation of more than one $G_{i/o/z}$ protein (Tso and Wong, 2000a; Tso and Wong, 2000b; Tso and Wong, 2001). Nonetheless, in experiments performed by another research group, G_z alone could substitute for $G_{i/o}$ to mediate adenylate cyclase

superactivation, although only a small effect was seen (Ozawa et al., 1999). The discrepancy in results between the studies might be due to differences in the level of over-expression of G_z in the two experiments. Together, the data seem to suggest that the parallel stimulation of more than one G_i, G_o or G_z protein is required for the chronic opioid induced superactivation of adenylate cyclase activity *in vitro*.

In the brain, μ opioid receptors have been reported to couple to G_{i2} (Garzon et al., 1997a), G_z (Garzon et al., 1997b; Garzon et al., 1997a; Garzon et al., 1998) and G_o (Jiang et al., 2001). There is evidence that the 'μ receptor', which morphine binds to, prefers G_z over G_{i2} (Garzon et al., 1998). High affinity binding of morphine to its receptor was significantly attenuated after pre-incubation of brain membranes with antibodies against $G\alpha_z$. In contrast, pre-treatment with $G\alpha_{i2}$ antibodies did not affect morphine binding to its receptor. When another preferential agonist at the μ receptor, [D-Ala^2,N-MePhe^4,Gly-ol^5]-enkephalin (DAMGO) was tested, there was a significant reduction in receptor binding affinity after pre-treatment with both antibodies (Garzon et al., 1998). Since antibody pre-treatment is assumed to prevent the G protein from interacting with its receptor, leading to a decrease in receptor affinity for its agonists, these data suggest that the 'morphine' receptor couples preferentially to G_z, while the 'DAMGO' receptor couples to both G_z and G_{i2} in the brain. Both the 'morphine' and 'DAMGO' receptors are likely to be opioid receptors of the μ subtype as the experiment was performed in the presence of the δ receptor antagonist, ICI-174,864, and the experimental conditions employed favour the detection of high affinity receptor binding states (Garzon et al., 1998). Although there is evidence for the existence of different pharmacological subtypes of μ receptors (Pasternak, 2001), only one μ receptor has ever been cloned (Kieffer, 1999). Receptor heterodimerization between opioid receptor subtypes (e.g μ-δ, δ-κ) can generate 'novel receptors' with distinct pharmacological profiles (Jordan and Devi, 1999) and G protein coupling preferences (George et al., 2000). Furthermore, multiple carboxyl terminal spliced variants of the μ receptor have been reported to exist (Pasternak, 2001), greatly increasing the potential of generating μ receptors with different pharmacological properties. The 'morphine' and 'DAMGO' receptors may therefore correspond to homogeneous or heterogeneous populations of these μ receptors, or μ-δ heteroreceptors.

Consistent with G_z playing a role in transducing the effects of morphine receptor activation in the brain, mice lacking the α subunit of G_z, have been observed to demonstrate either reduced supraspinal morphine analgesia (as measured by the 'hot plate' test) (Yang et al., 2000) or no difference in acute morphine analgesia but greater development of tolerance to the supraspinal analgesic effects of morphine after chronic treatment (Hendry et al., 2000). However, there were a few deficiencies in both of these studies. Firstly, mice of mixed genetic backgrounds were used and the hitchhiking of 'background genes' in the strains employed have been reported to be potentially capable of contributing to the analgesic effects of morphine (Lariviere et al., 2001). Secondly, basal nociceptive thresholds were not taken into account when analysing morphine analgesia. For instance, the control mice in Yang et al. (2000)'s paper have a longer baseline response latency than $G\alpha_z$ knockout mice. After taking into account this difference in response baseline, it appears that there may be little or no difference in morphine induced analgesia between mice of the two genotypes. Thirdly, gender differences in morphine analgesia (Cicero et al., 2002; Kepler et al., 1991; Krzanowska et al., 2002) have not been considered in both studies. In our earlier paper, we have used mice of mixed genders (same number of mice from each genotype). The gender of the mice was not reported in Yang et al. (2000). The mixing of mouse genders could mask any gender difference that might be present.

In this chapter, I have sought to confirm the involvement of $G\alpha_z$ in supraspinal morphine analgesia (as measured by the hot plate test) (Pastoriza et al., 1996) and tolerance by clarifying the issues discussed above and exploring whether the observed effect is dependent on the quantity of $G\alpha_z$ present. The finding of a gene dose relationship would further strengthen the hypothesis that $G\alpha_z$ plays an essential role in the development of morphine tolerance. I have also investigated some of the mechanisms that may explain the increased morphine tolerance observed in the $G\alpha_z$ knockout mouse. My results suggest that the augmented tolerance in the mutant mouse is not due to changes in morphine metabolism, and greater tolerance still develops in the absence of opportunities for associative learning. Besides showing increased analgesic tolerance, the $G\alpha_z$ knockout animals also demonstrated greater tolerance to the toxicity effects of morphine after chronic treatment. This suggests that the loss of $G\alpha_z$ may have a protective effect against drug over-dose among chronic morphine users. Finally, I have also investigated whether physical dependence on morphine is altered in these

mutant mice. The absence of $G\alpha_z$ is associated with a reduction in naloxone precipitated jumping behaviour, indicating that the signalling pathways mediated by $G\alpha_z$ are also involved in the expression of physical dependence.

3.2 Results

3.2.1 Basal nociceptive threshold and habituation to the hotplate.

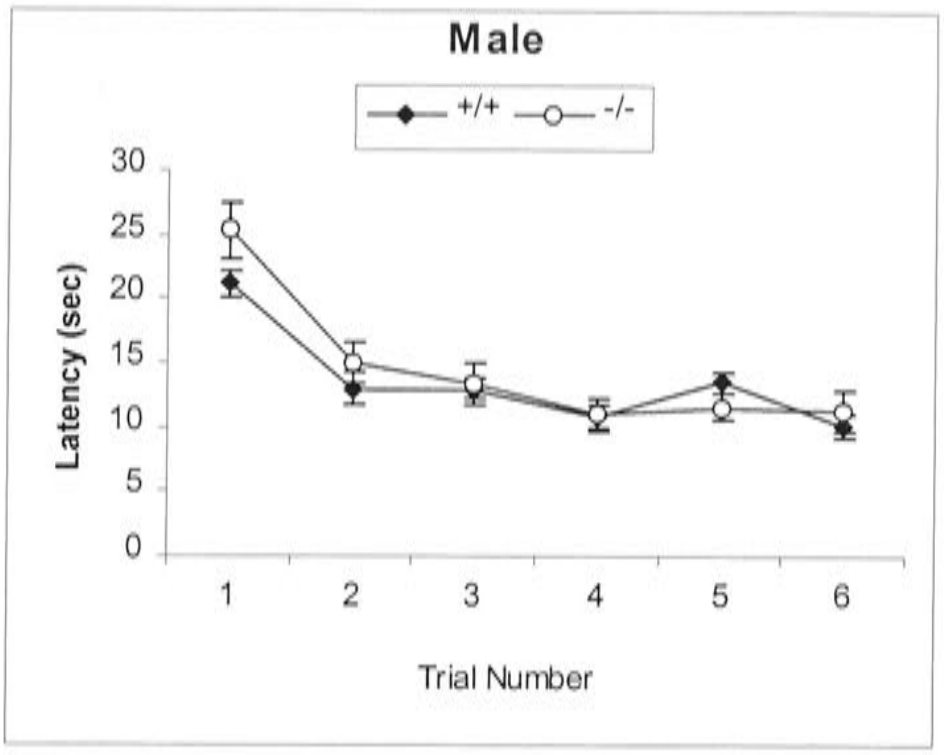
The baseline response latencies of male and female wildtype and $G\alpha_z$ knockout mice were examined in the drug free condition over 6 trials. Mice deficient in $G\alpha_z$ showed a slightly enhanced response latency on the first trial. This difference between mice of the two genotypes was abolished after the mice have habituated to the hotplate on subsequent trials (Figure 3.1). Therefore, the mice employed in the following experiments were always habituated to the hotplate for 3 trials before being tested for morphine analgesia using a cumulative morphine dosing regime (Sora et al., 1997). To compute the baseline response latency, the responses of each mouse from the second and third trials were averaged.

3.2.2 Acute morphine analgesia is altered in the $G\alpha_z$ knockout mouse.

Acute morphine analgesia was tested in drug free wildtype and $G\alpha_z$ knockout mice. The loss of $G\alpha_z$ produced a small gene dose dependent rightward shift in the morphine dose response curve in female mice (Genotype main effect: $F(2,25)=15.032$, $p<.001$), that was absent in male mice ($F(2,22)<1$, NS) (Figure 3.2a). Male $G\alpha_z$ knockout mice demonstrated a tendency of reduced morphine analgesia at low morphine doses, which disappeared at doses $\geq$ 35mg/kg.

3.2.3 Gene dose dependent alteration of morphine tolerance on the hotplate.

Next, I investigated whether $G\alpha_z$ might play a greater role in mediating the analgesic effects of morphine after tolerance development. Mice that have been treated with morphine for 3 days were tested again on the morning of the fourth day. A distinct difference was apparent in both male (Genotype main effect: $F(2,22)= 26.2$, $p<.001$)



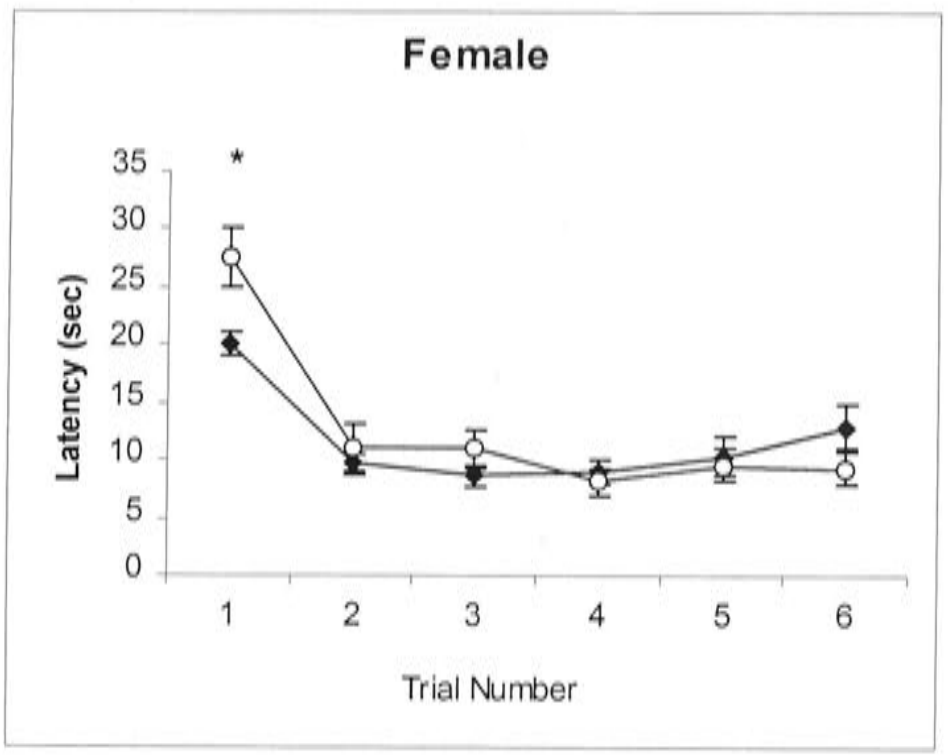


Figure 3.1: Basal nociceptive threshold and habituation to the hotplate in wildtype and $G\alpha_z$ knockout mice. The baseline response latencies of male (27 +/+, 27 -/-) and female (16 +/+, 16 -/-) wildtype and $G\alpha_z$ knockout mice were determined on the 52°C hotplate over six trials. Means ± S.E.M. are shown. * $p<.05$

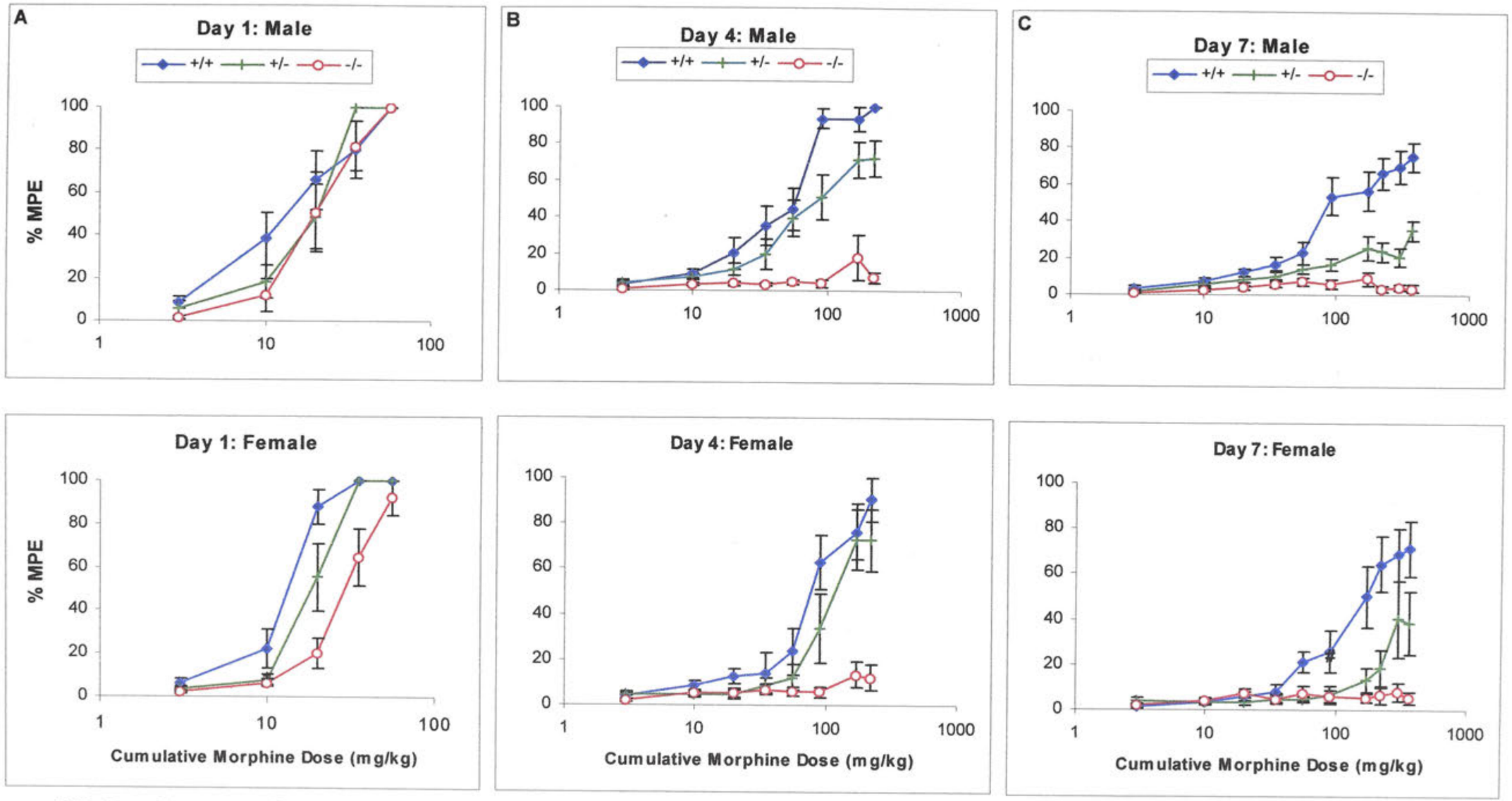


Figure 3.2: Development of morphine tolerance in the $G\alpha_z$ deficient mouse. Cumulative morphine dose response curves (on a logarithmic scale) were generated in male (9 +/+, 8 +/-. 8 -/-) and female (10 +/+. 8 +/-. 10 -/-) $G\alpha_z$ knockout (-/-), heterozygous (+/-) and wildtype (+/+) mice on the 52°C hotplate test. (A) Drug free mice treated acutely with morphine. (B) Mice that have been chronically treated with morphine for 3 days were tested on day 4. (C) The same mice had been chronically treated with morphine for 6 days and were tested on day 7. The values plotted are the means +/- SEM. The method used is described in Chapter 2.

and female ($F(2,25)=12.5$, $p<.001$) wildtype and $G\alpha_z$ knockout mice, where mice lacking $G\alpha_z$ appear to have stopped responding to morphine (Figure 3.2b). A further 3 days of morphine treatment caused wildtype and heterozygous mice to become more tolerant, with little effect on $G\alpha_z$ knockout mice, which were already very tolerant 3 days earlier. The heterozygous mice appeared to develop tolerance at a faster rate than wildtype mice (Figure 3.2c), producing an intermediate level of response that was significantly reduced compared to male (Genotype main effect: $F(1,15)= 24.1$, $p<.001$) and female ($F(1,16)= 7.183$, $p<.02$) wildtype mice.

3.2.4 Greater analgesic tolerance to morphine in $G\alpha_z$ knockout mice occurs in the absence of opportunities for behavioural learning.

In the behavioural protocol employed in our earlier paper (Hendry et al., 2000) and here, mice were habituated to the hotplate in earlier test sessions before showing a difference in their behavioural responses on subsequent days. Such habituation to the test environment during the period of morphine treatment has been reported to significantly decrease morphine induced analgesia compared to animals which have not been habituated (Gebhart and Mitchell, 1971; Gebhart and Mitchell, 1972; Milne and Gamble, 1989). To exclude the possibility that the observed behavioural difference reported above was due to a greater capacity of the $G\alpha_z$ knockout mouse to learn compensatory behavioural strategies associated with hot plate testing, additional experiments were performed where mice were tested for the first time on the hotplate after 6 days of chronic morphine treatment (these mice were not tested on days 1 and 4). Both male and female mice were used, with an equal number of both genotypes for each gender. Compared to drug-free mice (Figure 3.2a), the first exposure of chronically morphine treated mice to the hotplate reproduced the same altered morphine tolerance (Genotype main effect: $F(1,18)= 9.08$, $p<.01$), with $G\alpha_z$ knockout animals failing to respond to morphine doses up to 300 mg/kg and wildtype animals showing much less tolerance (Figure 3.3).

Furthermore, in the cumulative morphine dosing protocol, mice were injected with morphine every 20 minutes prior to analgesic testing on the hotplate. It may be argued that the increased tolerance that developed in the $G\alpha_z$ knockout mouse is associative in nature due to mutant mice learning the association between morphine injection and hotplate testing faster than wildtype mice. Associative tolerance to morphine involves

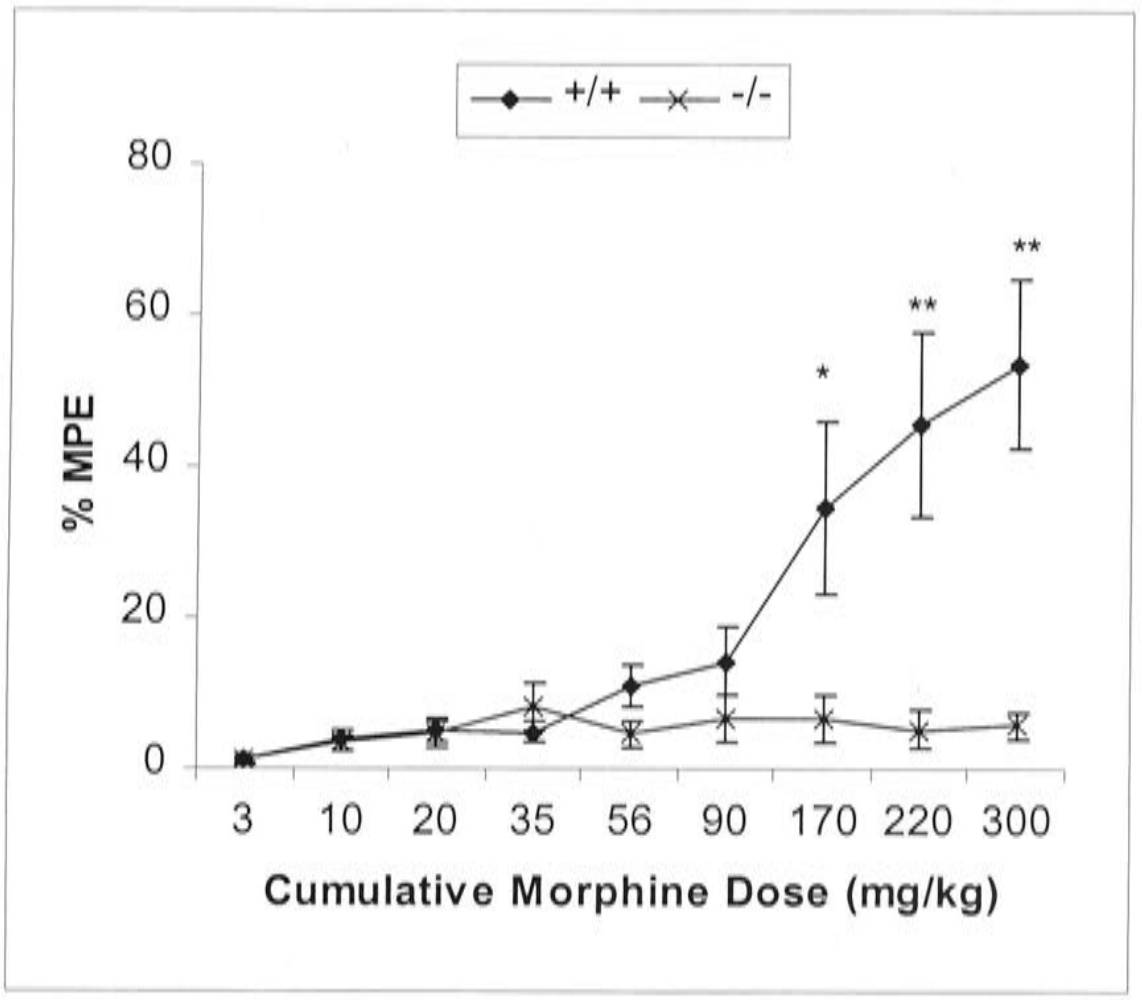


Figure 3.3: Altered morphine tolerance in the $G\alpha_z$ knockout mouse occurs in the absence of behavioral habituation. Ten mice of each genotype (6 males, 4 females) were chronically treated with morphine for six days (starting from a total cumulative dose of 100 mg/kg on day 1 to 600 mg/kg on day 6). On day 7, they were exposed to the 52°C hot plate for the first time and the response latency of each mouse to a cumulatively increasing dose of morphine was recorded. * $p<.05$, ** $p<.01$

the participation of neuronal circuitry in the amygdala (Mitchell et al., 2000), where $G\alpha_z$ has been found (Glick et al., 1998; Wang et al., 1998). To investigate whether the $G\alpha_z$ knockout mouse would still develop greater tolerance to morphine in the absence of opportunities for associative learning, wildtype and mutant mice were implanted with morphine pellets, and tested on the hotplate 2, 24, 48, 72 and 96 hours after the implant. Morphine lost its analgesic effects in the $G\alpha_z$ knockout mouse about 48 hours after the implant, compared to 96 hours in wildtype mice (Figure 3.4) (Genotype main effect: $F(1,11)= 5.44$, $p<.05$). Taken together, these results suggest that the increased analgesic tolerance to morphine that developed in the $G\alpha_z$ knockout mouse is not a result of behavioural learning.

3.2.5 Gene dose dependent morphine lethality in tolerant mice

Lethality from morphine over-dose is known to be mediated through the μ opioid receptor (Loh et al., 1998; Sora et al., 2001a). To investigate whether the $G\alpha_z$ knockout mouse were indeed more tolerant to high doses of morphine, drug-free and chronically morphine treated wildtype and $G\alpha_z$ knockout mice were administered an over-dose of morphine. Most mice entered a convulsive state and died within the first 4 hours of injection. However, morphine treated $G\alpha_z$ knockout mice were found to be capable of tolerating higher morphine doses compared to similarly treated wildtype and heterozygous mice (Table 3.1). Whereas the median lethal dose (LD50) of chronically treated wildtype mice was less than 700mg/kg, heterozygous mice showed a LD50 of about 750mg/kg, and chronically treated $G\alpha_z$ knockout mice had a LD50 greater than 800mg/kg. Drug-free wildtype and $G\alpha_z$ knockout mice had a LD50 of 400mg/kg, similar to values reported by Loh et al. (1998).

3.2.6 Wildtype and $G\alpha_z$ knockout mice show similar morphine pharmacokinetics.

The liver plays an important role in morphine metabolism (Aasmundstad et al., 1993) and $G\alpha_z$ has been reported to be expressed in the liver (Spicher et al., 1988). Therefore, I examined whether pharmacokinetic differences could account for the development of greater morphine tolerance in the $G\alpha_z$ knockout mouse. Serum levels of morphine and its major metabolites, M3G and Nor-M3G in male and female wildtype and $G\alpha_z$ knockout mice on day 7 of morphine treatment were measured. Despite the $G\alpha_z$ knockout animals

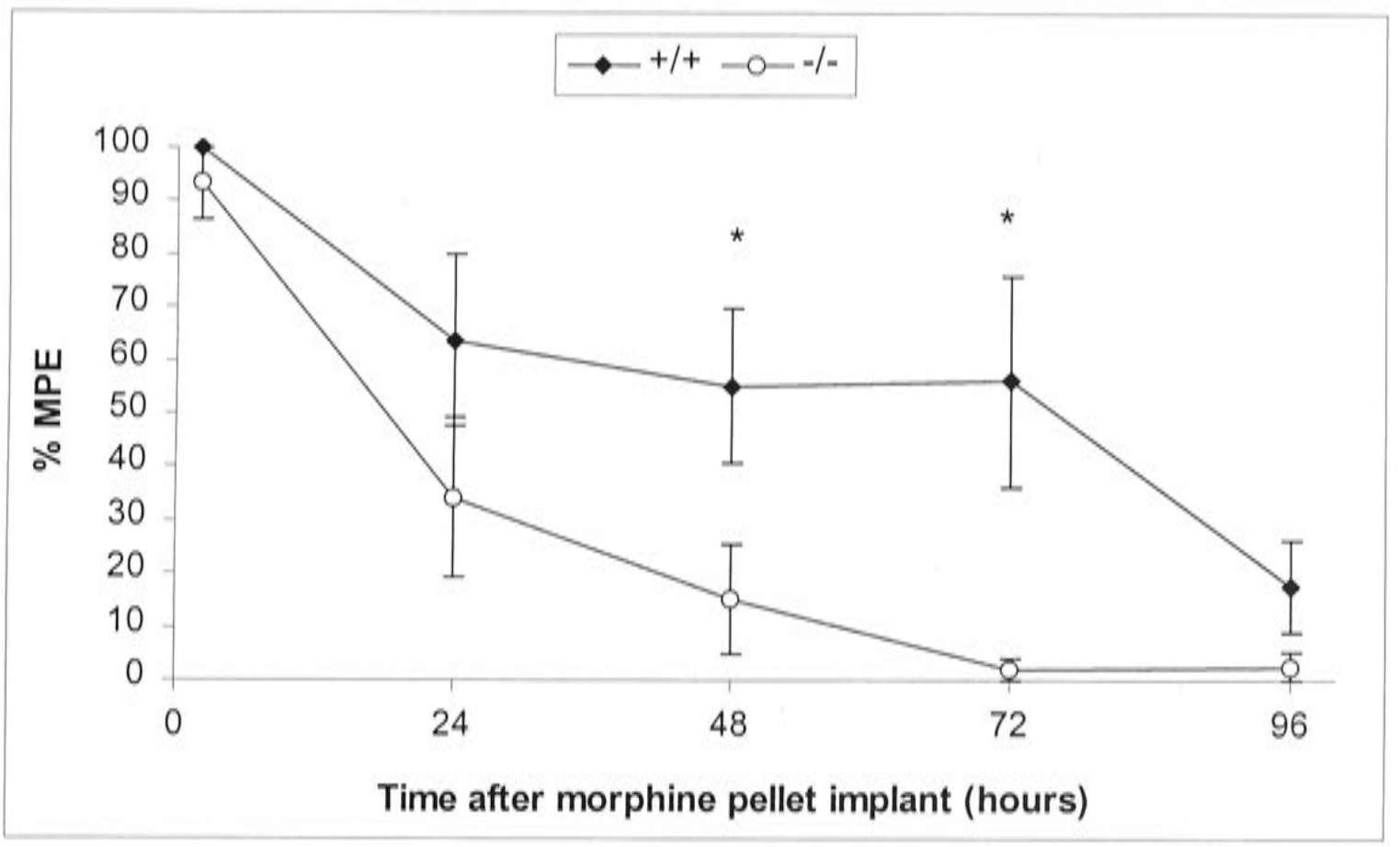


Figure 3.4: Non-associative tolerance to the analgesic effects of morphine is enhanced in the $G\alpha_z$ knockout mouse. Male wildtype and $G\alpha_z$ knockout mice (6+/+, 7-/-) were implanted with 75mg morphine pellets, and tested on the hotplate 2, 24, 48, 72 and 96 hours after the implant. The mean %MPE ± S.E.M. are shown. Similar data were obtained for two other groups of male and female mice tested for up to 48 hours after the implant. * $p<.05$

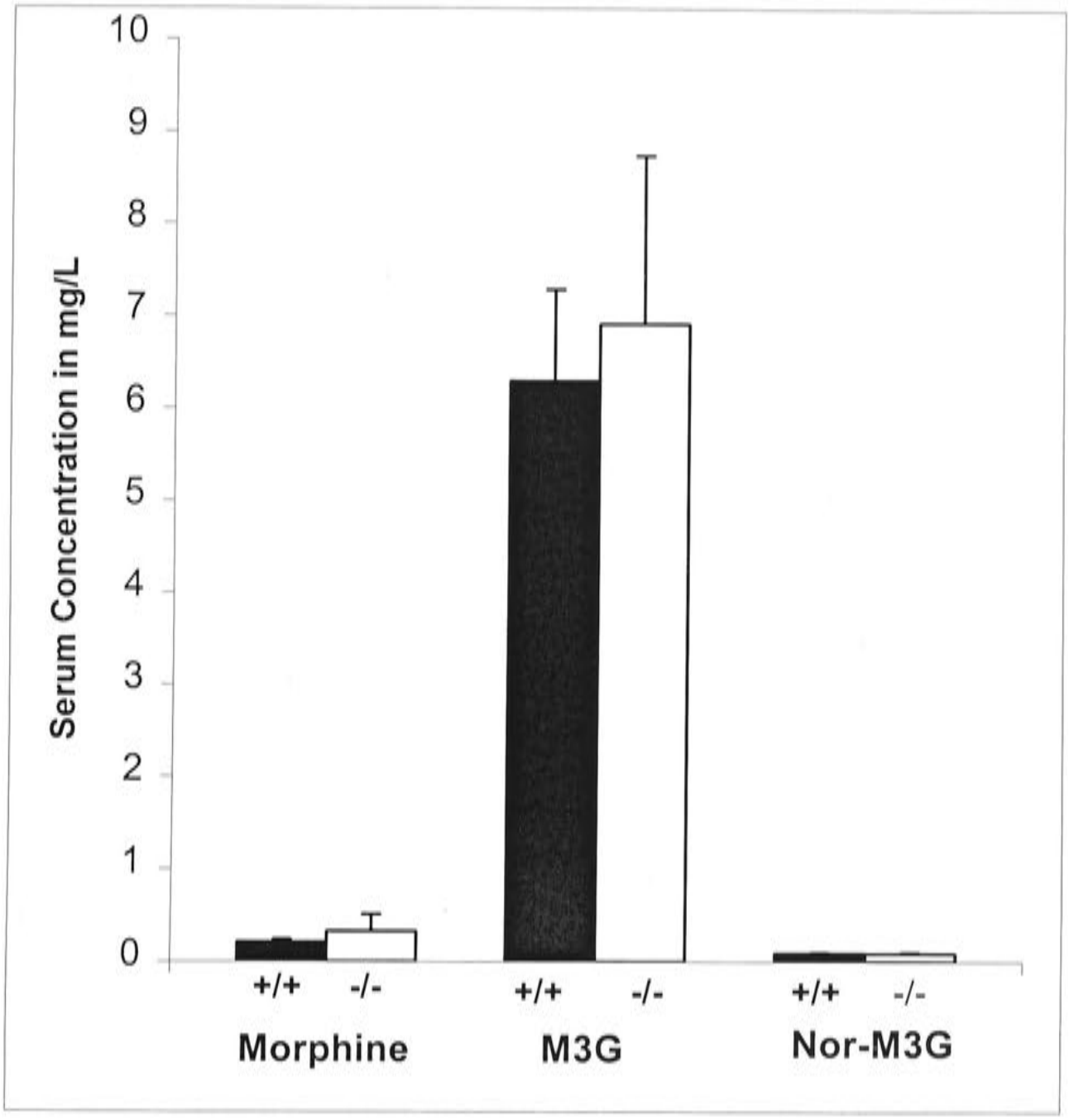


Figure 3.5: Serum morphine, morphine-3-glucuronide (M3G) and nor-morphine-3-glucuronide (nor-M3G) levels in tolerant $G\alpha_z$ knockout (-/-) and wildtype mice (+/+). Six mice of each genotype were chronically treated with morphine for 6 days (starting from a total cumulative dose of 100 mg/kg on day 1 to 600 mg/kg on day 6). On day 7, mice were administered 50mg/kg of morphine and 2 hours later, mice were anaesthetized and trunk blood was collected from the jugular vein. Two other experiments (one performed on tolerant male mice and the other on tolerant female mice) sacrificed at different time intervals after morphine administration produced similar levels of morphine and M3G between wildtype and $G\alpha_z$ knockout mice.

Table 3.1: Percentage of morphine tolerant mice that survive various doses of morphine. Figures in parentheses indicate the actual number of mice tested that survived the particular morphine dose.

Morphine Dose	+/+	+/-	-/-
900mg/kg	0% (0/2)	0% (0/2)	33.3% (2/6)
800mg/kg	13.0% (3/23)	42.9% (6/14)	76.5% (13/17)
700mg/kg	33.3% (3/9)	60% (3/5)	100% (8/8)

showing greater tolerance, there was no significant difference in the levels of morphine or its metabolites in the serum (Figure 3.5).

3.2.7 Alteration of physical dependence on morphine in the $G\alpha_z$ knockout mouse

Morphine tolerance and physical dependence have been suggested to share a common underlying mechanism (Way et al., 1969). Since the $G\alpha_z$ knockout mouse show greater tolerance to morphine, it is possible that they may also be more dependent physically. The degree of physical dependence is usually measured based on the severity of morphine withdrawal signs, after withdrawal has been precipitated in tolerant animals by treating with the opioid antagonist, naloxone. One of the most prominent sign of morphine withdrawal in rodents is naloxone precipitated jumping behaviour, whose severity has previously been found to correlate with the degree of morphine tolerance (Way et al., 1969). Contrary to expectations, I observed a gene dose dependent reduction of naloxone precipitated jumping in both male (Kruskal-Wallis ANOVA by genotype, n= 54, χ^2 = 22.5, p<.001) and female (n =44, χ^2 = 17.4, p<.001) $G\alpha_z$ knockout mice (Figure 3.6a). However, there was no consistent gene dose relationship across genders in the other withdrawal signs, wet dog shake (Figure 3.6b) and naloxone induced weight loss (Figure 3.6d). For naloxone elicited depression of locomotor activity (Figure 3.6c), there appears to be a gene dose dependent trend for the $G\alpha_z$ knockout mouse to be more severely affected. However, the relationship was not statistically significant in both male (χ^2= 4.58, p=.10) and female (χ^2= 4.60, p=.10) $G\alpha_z$ knockout mice.

3.2.8 Cyclic AMP levels in saline and chronically morphine treated mice

One cellular hallmark of morphine withdrawal is the compensatory upregulation of cyclic AMP levels by an opioid antagonist (Finn and Whistler, 2001). Cyclic AMP levels were measured in the striatum and periaqueductal gray of chronic morphine or saline treated mice, 20 minutes after the administration of naloxone. These two brain regions were chosen because they have previously been known to contain opioid sensitive neurons (Gutstein et al., 1998; Petruzzi et al., 1997) and $G\alpha_z$ (Garzon et al., 1997b; Garzon et al., 1997a; Glick et al., 1998; Hinton et al., 1990; Wang et al., 1998). The level of cyclic AMP in the striatum of morphine tolerant $G\alpha_z$ knockout mice was

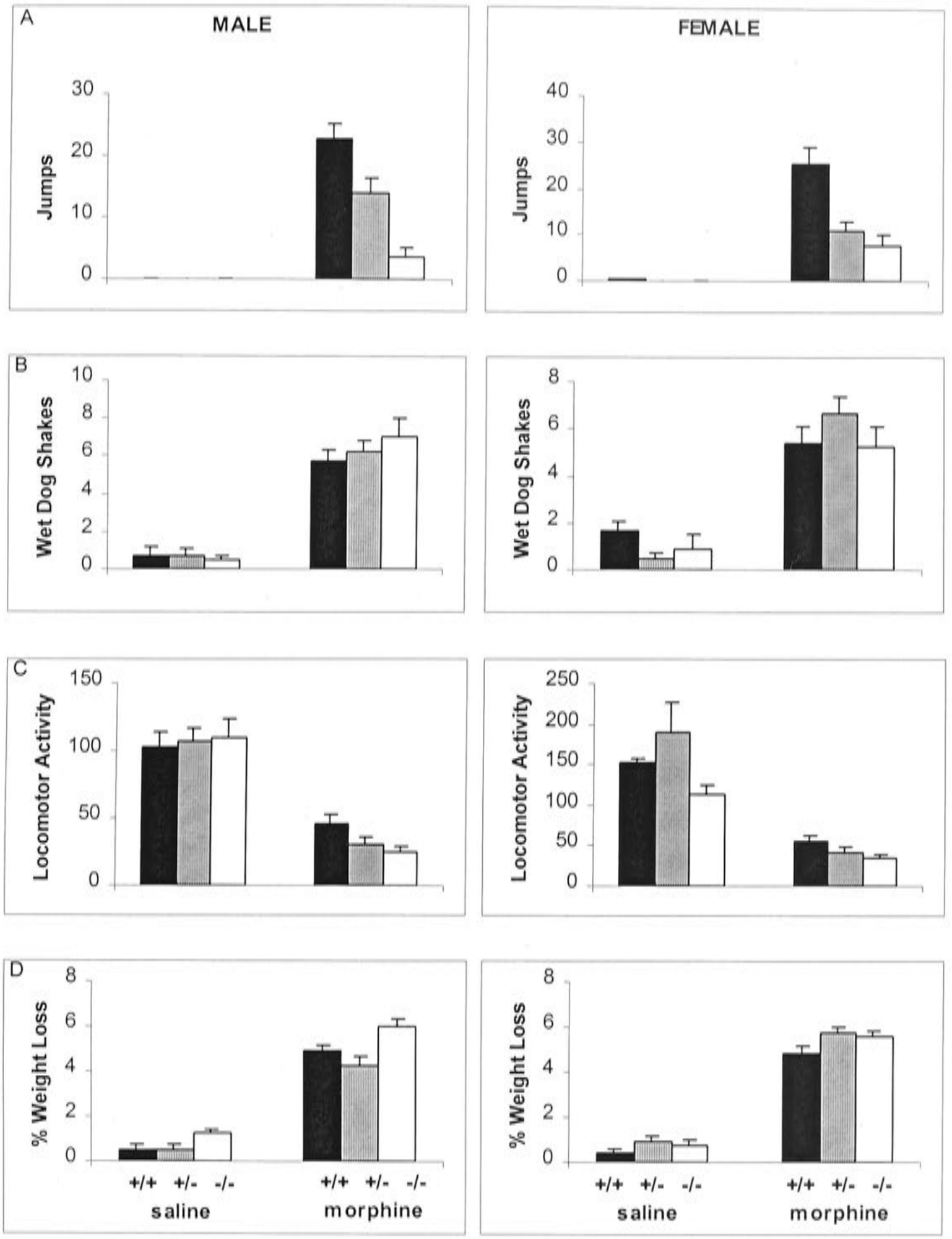


Figure 3.6: Alteration of physical dependence on morphine in the $G\alpha_z$ knockout mouse. Morphine tolerant (male: 21 +/+, 17 +/-, 16 -/-; female: 17 +/+, 13 +/-, 14 -/-) or saline treated (male: 8 +/+, 13 +/-, 11 -/-; female: 8 +/+, 9 +/-, 9 -/-) mice were given a priming dose of either 50mg/kg morphine or saline after 7 days of treatment. Two hours later, the mice were weighed, and abrupt withdrawal was precipitated using 4mg/kg naloxone. Immediately, each mouse was placed into a large observation cage, and two observers who were blind to the mice's genotype and drug treatment condition counted (A) the number of jumps, (B) wet dog shakes and (C) line crossings (locomotor activity) made by each mouse within a 15 minutes time period. The correlations between the scores of the two observers were 0.99 for jumps, 0.81 for wet dog shakes, and 0.98 for line crossings. The mice were weighed again 45 minutes later, and (D) the percentage weight loss was computed for each mouse. Means ± S.E.M. are shown.

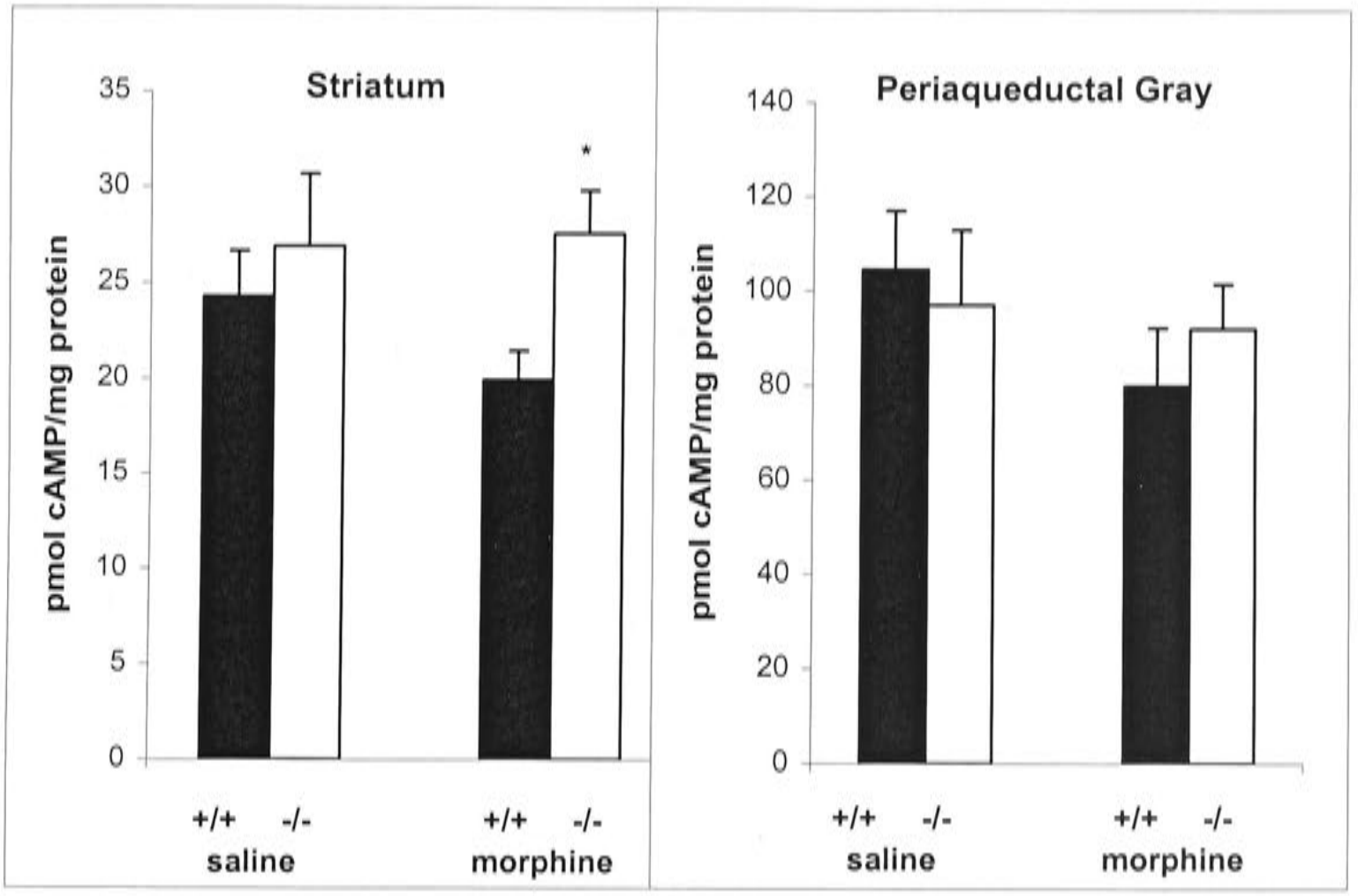


Figure 3.7: Cyclic AMP levels in the striatum and periaqueductal gray of chronically morphine and saline treated mice. Male wildtype and $G\alpha_z$ knockout mice were chronically treated with morphine (12 +/+, 12 -/-) or saline (18 +/+, 6 -/-) according to the protocol described in Chapter 2, followed by an intraperitoneal injection of 4mg/kg naloxone HCl, 2 hours after the last treatment. The mice were sacrificed 20 minutes later by rapid decapitation and the head was immediately irradiated in a 1kW microwave for 3 seconds to inactivate phosphodiesterases. The striatum and periaqueductal gray (PAG) were dissected on ice, and cyclic AMP was extracted using ethanol. Tissue levels of cyclic AMP and protein were measured using kits that were commercially available. Means ± S.E.M. values are shown. Comparison between morphine treated +/+ and -/- mice: * $p<.05$.

greater than tolerant wildtype mice ($t(22)= 2.81$, $p<.02$) (Figure 3.7). However, none of the levels measured in morphine treated mice was above that of saline treated animals.

3.2.9 Gene dose dependent expression of $G\alpha_z$ protein in the brain of the mouse.

The gene dose dependent effect on morphine tolerance and naloxone precipitated jumping suggest that the level of expression of the $G\alpha_z$ protein depends on gene dosage. The levels of $G\alpha_z$ protein in the brains of male wildtype, heterozygous and $G\alpha_z$ knockout mice were analysed on western blots. The result showed that the level of $G\alpha_z$ protein in the brains from heterozygous mice was about $41.6 \pm 4.1\%$ ($n=4$) of that from wildtype mice (Figure 3.8).

3.2.10 Lack of compensation by other G protein α subunits in drug free and morphine tolerant mouse brains.

The levels of expression of other G protein α subunits were determined to assess whether the observed phenotypes could be due to compensation by other G proteins. In both naïve and morphine tolerant mouse brains, there was no detectable change in the level of expression of $G\alpha_i$, $G\alpha_o$, $G\alpha_q$ and $G\alpha_s$ (Figure 3.9).

3.3 Discussion

The clinical importance of morphine as an analgesic has made understanding the mechanisms underlying morphine analgesia, tolerance and dependence one of the key goals in modern pain research. This goal has been facilitated by the advent of gene knockout technology, which allowed mice with defined lesions to specific molecular targets along the opioid signal transduction pathway to be made. Using this approach, the μ opioid receptor has been identified to be essential for morphine induced analgesia and physical dependence (Loh et al., 1998; Matthes et al., 1996; Sora et al., 1997) and the δ receptor for the development of analgesic tolerance (Zhu et al., 1999b). Both of these receptors have been shown to couple to $G\alpha_z$ *in vivo* (Garzon et al., 1997a) and *in vitro* (Ho and Wong, 2001). Previous work employing the antisense oligonucleotide approach to examine the role of $G\alpha_z$ in acute supraspinal morphine analgesia have yielded varying results, with one report demonstrating a significant attenuation of

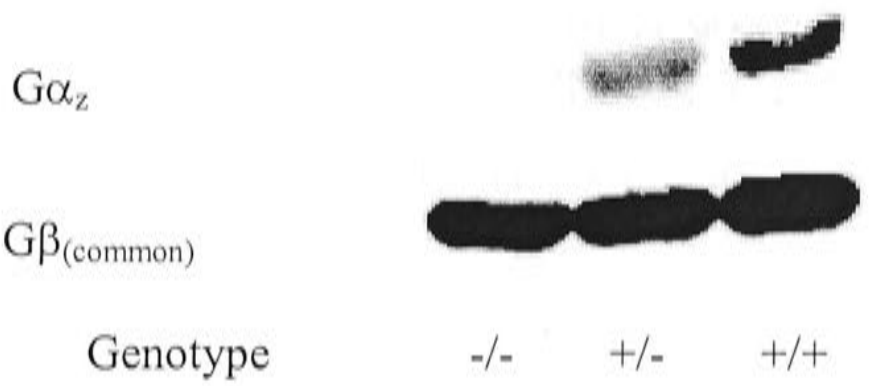


Figure 3.8: Gene dose dependent expression of $G\alpha_z$ proteins in wildtype, heterozygous, and knockout mouse brains. The proteins from the brains of four male mice of each genotype were analysed by SDS- polyacrylamide gel electrophoresis, electro-transferred onto nitrocellulose, and blotted with a $G\alpha_z$ specific antibody. The blot was subsequently re-probed with a $G\beta_{(common)}$ antibody as a gel loading control.

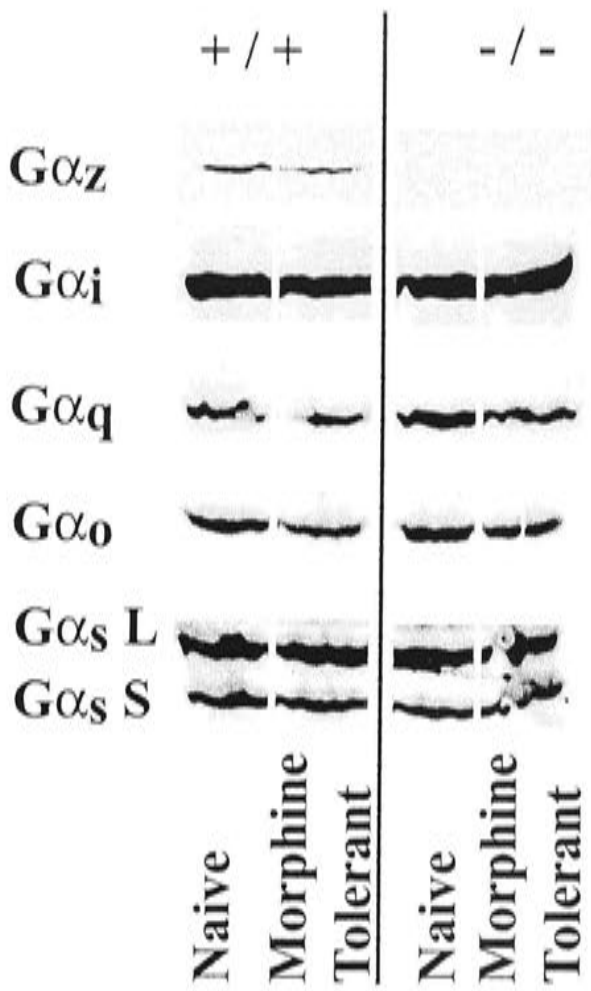


Figure 3.9: Levels of $G\alpha_z$, $G\alpha_i$, $G\alpha_q$, $G\alpha_o$, $G\alpha_{s(long)}$ and $G\alpha_{s(short)}$ in the brains from naïve and morphine tolerant wildtype and $G\alpha_z$ knockout mice. The proteins from the brains of naïve and morphine tolerant wildtype and $G\alpha_z$ knockout mice were analysed by SDS- polyacrylamide gel electrophoresis, electro-transferred onto nitrocellulose, and blotted with antibodies specific for $G\alpha_z$, $G\alpha_i$, $G\alpha_q$, $G\alpha_o$, and $G\alpha_s$.

supraspinal morphine analgesia after loss of $G\alpha_z$ (Sanchez-Blazquez et al., 1995), and another showing little effect (Standifer et al., 1996). The latter, however, has employed an oligonucleotide against the human $G\alpha_z$ gene sequence, which may not be effective against mouse $G\alpha_z$ (see Chapter 1, section 1.4.1). I have therefore used the $G\alpha_z$ knockout mouse generated in my laboratory to investigate the involvement of $G\alpha_z$ in morphine analgesia in greater detail.

The earlier studies by Yang et al. (2000) and my laboratory have employed $G\alpha_z$ knockout mice of mixed genders and genetic backgrounds, and morphine analgesia was measured without correcting for differences in baseline response latencies. While the baseline response latencies of the $G\alpha_z$ knockout mice used by Yang et al. (2000) were shorter compared to their control mice, our mutant mice demonstrate a longer latency relative to wildtype mice on the first trial. The reason for this difference is unclear. Since our mice were in a pure C57BL/6 genetic background and the mice used by Yang et al. (2000) were from a mixed C57BL/6:129/Sv:SJL background (Ioffe et al., 1995), the difference could be due to genes from either the 129/Sv or SJL backgrounds or both. Alternatively, differences in experimental procedure (Yang et al. (2000) used a 58°C hotplate) might have also contributed to the difference.

When acute morphine analgesia was examined, a gender difference was apparent, with female $G\alpha_z$ knockout mice demonstrating a gene dose related decrease in morphine analgesia, and little difference among male mice. Gender variations in morphine antinociception have generally been attributed to differences in the organization of male and female brains (Cicero et al., 2002; Krzanowska et al., 2002). The periaqueductal gray, an area of the brain involved in the production of morphine antinociception (Behbehani et al., 1990; Smith et al., 1988), contains $G\alpha_z$ (Garzon et al., 1997b; Garzon et al., 1997a), and there is evidence that its organization may differ between the genders (Bandler and Shipley, 1994; Schwartz-Giblin and McCarthy, 1995). It is possible that the relative importance of the G_z signalling pathway in mediating the acute analgesic effects of morphine may become altered as a result of these circuitry changes, resulting in the small gender difference observed here.

On the whole, the finding that acute morphine analgesia is only slightly diminished in the $G\alpha_z$ knockout mouse support data reported in the literature that the acute analgesic

effects of morphine are transduced mainly through other G proteins, notably G_{i2} and possibly G_o (Raffa et al., 1994; Standifer et al., 1996). On the other hand, it is also possible that G_z usually plays a greater role in acute morphine analgesia (Sanchez-Blazquez et al., 1995; Sanchez-Blazquez et al., 2001) and the current small difference between wildtype and $G\alpha_z$ knockout mice is due to compensatory changes occurring in the antinociceptive pathways of the $G\alpha_z$ knockout animal. Although such an explanation cannot be ruled out, I have excluded compensatory changes in the levels of other G proteins in the brains of drug free and morphine tolerant $G\alpha_z$ knockout mice as a possible explanation.

Following chronic morphine treatment, the behavioural difference between $G\alpha_z$ knockout mice and wildtype controls in response to morphine becomes very distinct and exhibits a gene dose relationship. This confirms previous observations from my laboratory that $G\alpha_z$ is important for the development of tolerance to the supraspinal analgesic effects of morphine (Hendry et al., 2000). I have further demonstrated that this effect is not due to pharmacokinetic or behavioural factors. The excitatory morphine metabolites, M3G and nor-M3G, which have been postulated to play a role in the development of morphine tolerance (Smith and Smith, 1995; Smith and Smith, 1998), are present in similar concentrations in the sera of tolerant wildtype and $G\alpha_z$ knockout mice. The lack of opportunity for behavioural learning also did not prevent the $G\alpha_z$ knockout mouse from showing greater tolerance to the analgesic effects of morphine on the hotplate test. These results imply that the mechanism that contributes to the increased morphine tolerance in the $G\alpha_z$ knockout mouse differs from those involved in the production of pharmacokinetic or behavioural tolerance to the analgesic effects of morphine.

Besides analgesia, the μ opioid receptor has also been shown to be responsible for the toxicity effects of morphine (Loh et al., 1998; Sora et al., 2001a). Consistent with the observation of greater tolerance to supraspinal morphine analgesia in the $G\alpha_z$ knockout mouse, I found a similar gene dose dependent development of tolerance to the lethality effects of morphine. Therefore, the loss of $G\alpha_z$ not only produces greater tolerance to the supraspinal analgesic effects of morphine, but also to its lethality effects, which presumably is also mediated through supraspinal brainstem mechanisms (Florez and Hurle, 1993; Loh et al., 1998).

$G\alpha_z$, $G\alpha_{i2}$ and $G\alpha_o$ have all been shown to be capable of coupling to μ receptors in the brain (Garzon et al., 1997b; Garzon et al., 1997a; Garzon et al., 1998; Jiang et al., 2001). The data presented here suggests that other heterotrimeric G protein α subunits are not able to take over the signalling functions of $G\alpha_z$ after the development of morphine tolerance. There might therefore be distinct subtypes of the μ receptor which prefer binding to different G proteins (Garzon et al., 1998). Recent evidence suggests that there are multiple splice variants of the μ receptor. These splice variants differ in their tissue distributions and from one another in their carboxyl terminal amino acids (Abbadie et al., 2000; Pan et al., 1999), a region of the receptor apparently important in determining G protein specificity (Namba et al., 1993). Some of these splice variants have also been found to exhibit different agonist induced desensitization properties (Abbadie and Pasternak, 2001; Koch et al., 2001; Zimprich et al., 1995). Receptor desensitization is an important mechanism thought to explain morphine tolerance (Bohn et al., 2000). Thus, it is possible that opioid receptors that preferentially couple to $G\alpha_z$ have different desensitization properties compared to receptors that prefer other G protein α subunits.

The heteromeric form of the μ and δ receptor is another possible candidate of an opioid receptor that might couple $G\alpha_z$ to transduce the analgesic effects of morphine after tolerance development. $G\alpha_z$ is the only pertussis toxin resistant member of the $G\alpha_i$ protein family (Ho and Wong, 2001). Using pertussis toxin as a tool, George et al. (2000) found pertussis toxin treatment completely abolished the high affinity binding of [D-Ala^2,N-Me-Phe^4, Gly^5-ol]-enkephalin (DAMGO) and [D-Pen^2,D-Pen^5]-enkephalin (DPDPE) to the homomeric forms of the μ and δ receptors respectively. However, pertussis toxin did not affect high affinity binding of DAMGO or DPDPE to the μ - δ heteromer (George et al., 2000). This suggests that homomeric μ and δ receptors are coupled to pertussis toxin sensitive G proteins, whereas the μ-δ heteromer is coupled to a pertussis toxin insensitive G protein. Furthermore, it was found that the μ-δ heteromer mediates pertussis toxin insensitive inhibition of adenylate cyclase (George et al., 2000), a property apparently unique to $G\alpha_z$ (Ho and Wong, 2001). Interestingly, the μ-δ heteromer was also resistant to receptor desensitization after chronic opioid exposure (George et al., 2000). These properties of the μ-δ heteromer are consistent with that of a receptor that would couple to $G\alpha_z$ to transduce the analgesic effects of morphine in tolerant animals.

Since the morphine receptor is potentially capable of coupling to a few other G proteins (Sanchez-Blazquez et al., 1995), an alternative explanation of our data may be that the loss

of any one of these G proteins will produce greater morphine tolerance. This explanation implies that in the tolerant state, the pool of G proteins that is available for coupling to receptors may be severely limited, such that there is no G protein remaining to compensate for the loss of any one of the other G proteins. Such an explanation, however, is made less likely by the finding of a substantial functional receptor reserve in morphine tolerant mice on the analgesic assay (Sora et al., 2001a), suggesting this is normally not the limiting factor in tolerant animals. As there is no difference in the total number of morphine receptors between tolerant wildtype and $G\alpha_z$ knockout mice (Hendry et al., 2000), this raises the question whether the identity of the G proteins that couple to the morphine receptor in the tolerant state could be different from the population of G proteins that couple to the receptor in the drug free state. The data presented here suggest that other G proteins (notably $G\alpha_{i2}$ and $G\alpha_o$, which are present in much larger quantities than $G\alpha_z$ in the brain), are not capable of compensating for $G\alpha_z$ in the tolerant animal. Although disruptions to the functionality of G_z coupled opioid receptors is the most likely explanation of the hypertolerance phenotype of $G\alpha_z$ deficient animals reported here, it is also possible that the phenotype was caused by changes in the functions of cells up or downstream of those that have opioid receptors, or that the development of animals lacking $G\alpha_z$ has been altered in such a way that morphine tolerance is more readily expressed.

Despite the observation of increased morphine tolerance, naloxone precipitated jumping, a prominent morphine withdrawal sign in rodents, is significantly attenuated in $G\alpha_z$ knockout mice. This result is in direct contradiction to that obtained by Way et al. (1969), where a linear positive correlation between morphine tolerance and naloxone precipitated jumping was reported. Nonetheless, the other major signs of morphine withdrawal (wet dog shakes, weight loss) are not significantly affected by the absence of $G\alpha_z$. This indicates a dissociation of not only between morphine tolerance and dependence, but also between the different symptoms of dependence. Such dissociations have recently been observed in a number of different genetically modified mice. For instance, β arrestin-2 knockout mice do not develop morphine tolerance, but they still become physically dependent (Bohn et al., 2000). Protein kinase C-γ mutant mice were less tolerant to morphine, but exhibited significantly more naloxone precipitated jumping behaviour. However, the other major withdrawal sign reported by the authors, naloxone induced weight loss, was significantly reduced (Zeitz et al., 2001). In substance P receptor knockout mice, naloxone precipitated jumping was significantly

decreased, but the other withdrawal signs were unchanged (Murtra et al., 2000). These dissociations suggest that morphine withdrawal is not a unitary phenomenon (Calvino et al., 1979). Rather, it appears to be an ensemble of signs, which reflects the consequences of activation of different neuronal pathways by naloxone. The impairment of naloxone precipitated jumping in the $G\alpha_z$ knockout mouse, may therefore result from a decrease in functioning of one of these pathways. Since $G\alpha_z$ can potentially couple to a number of receptors along the neuronal pathway that modulates naloxone precipitated jumping behaviour, it is not possible to pinpoint the particular receptor(s) whose function(s) has been affected. Probable candidates include the substance P receptor (Murtra et al., 2000; Ueda et al., 1987) and the $\alpha 2$ adrenergic receptor (Taylor et al., 1988). The substance P receptor can couple to G_z *in vitro* (Ho and Wong, 1998), while the $\alpha 2$ adrenergic receptor has been shown to couple to G_z both *in vitro* (Ho and Wong, 1998) and *in vivo* (Yang et al., 2000). Additionally, serotonergic and dopaminergic neurotransmission may also exert regulatory influences on naloxone precipitated jumping behaviour (El Kadi and Sharif, 1995; Schulz et al., 1978; Schulz and Herz, 1977) (Caille et al., 2002). There is evidence that some serotonin and dopamine receptors can couple to G_z *in vitro* (Ho and Wong, 1998).

When the level of cyclic AMP was measured in the periaqueductal gray and striatum of animals undergoing naloxone precipitated withdrawal, the level of cyclic AMP in chronically morphine treated animals undergoing withdrawal was not above that of saline treated animals. This suggests that the supersensitization of adenylate cyclase activity that is frequently observed in cultured cells (Finn and Whistler, 2001; Ozawa et al., 1999; Tso et al., 2000; Tso and Wong, 2000a; Tso and Wong, 2000b; Tso and Wong, 2001) does not occur in most neurons in these two brain regions. Alternatively, it is possible that the adenylate cyclases present in morphine tolerant tissues need to be stimulated with either forskolin or $G\alpha_s$ (Avidor-Reiss et al., 1997; Hanoune and Defer, 2001) before a compensatory increase in cyclic AMP levels above saline or untreated[1] tissues can be observed (Duman et al., 1988; Terwilliger et al., 1991; Van Vliet et al., 1991). However, it is interesting that the level of cyclic AMP in the striatum of tolerant $G\alpha_z$ knockout mice undergoing withdrawal is higher than that of tolerant wildtype mice. The striatum is rich in opioid receptors (Petruzzi et al., 1997), and is known to be one of the brain regions that mediate the locomotor effects of morphine (Stevens et al., 1986).

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[1] Untreated tissues have approximately the same level of cyclic AMP as saline treated tissues (data not shown).

The present observation of a higher level of cyclic AMP in the striatum of withdrawn $G\alpha_z$ knockout mice chronically treated with morphine may possibly suggest that most striatal $G\alpha_z$ knockout neurons are more withdrawn compared to striatal wildtype neurons. Phenotypically, this may relate to the trend for greater suppression of locomotor activity in these mice during naloxone precipitated withdrawal. Nonetheless, none of the withdrawal behaviours in the $G\alpha_z$ knockout animals demonstrate a clear increase in intensity although the animals are very tolerant to morphine. This indicates a dissociation between morphine tolerance and physical dependence in these animals.

In summary, I have shown that G_z plays an important role in mediating the supraspinal analgesic and lethality effects of morphine after tolerance development. The greater morphine tolerance that developed in the $G\alpha_z$ knockout animals were not due to pharmacokinetic or behavioural mechanisms. I have also demonstrated a gene dose dependent decrease in naloxone precipitated jumping in the $G\alpha_z$ knockout mouse. My data provides strong support for a dissociation between the different signs of physical dependence, indicating that morphine withdrawal is not a unitary phenomenon. It is therefore very important when measuring the degree of physical dependence, to consider a range of different withdrawal signs. In addition, the dissociation between morphine tolerance and naloxone precipitated jumping suggests that different G_z signalling pathways are involved in mediating the effects of the loss of $G\alpha_z$ on morphine tolerance and physical dependence. Clinically, it may be possible to selectively affect these separate pathways to prevent the development of morphine tolerance and physical dependence in human patients. Future investigations using the $G\alpha_z$ knockout mouse as an animal model should provide useful insights about the mechanisms underlying morphine tolerance and physical dependence.

Chapter 4

Alteration of morphine induced locomotor activation in the $G\alpha_z$ knockout mouse

4.1 Introduction

Morphine binds to cell surface opioid receptors to produce analgesia (Matthes et al., 1996), immunosuppression (Gaveriaux-Ruff et al., 1998), hypothalamic-pituitary axis stimulation (Roy et al., 2001), and locomotor activation in rodents (Sora et al., 2001a). Opioid receptors are G protein coupled receptors, which consist of three subtypes, designated μ, δ and κ. Although morphine can bind to all three receptor subtypes, it has the highest affinity for the μ receptor (Goldstein and Naidu, 1989; Raynor et al., 1994). Target inactivation of the μ receptor shows that all of the above effects are mediated by the μ receptor in mice (Becker et al., 2000; Gaveriaux-Ruff et al., 1998; Matthes et al., 1996; Roy et al., 2001; Sora et al., 2001a; Tian et al., 1997). Like other opioid receptors, the μ receptor couples to G proteins from the inhibitory GTP binding protein family (Standifer and Pasternak, 1997). Available evidence suggests that the μ receptor can potentially couple to a number of these G proteins, including G_{i2} (Garzon et al., 1997a), G_z (Garzon et al., 1997b; Garzon et al., 1997a; Garzon et al., 1998) and G_o (Jiang et al., 2001). However, less is known about the specific G protein(s) that mediate each of the many distinct effects of morphine *in vivo*.

There are three mechanisms whereby morphine stimulates locomotor activity in rodents, each of which may involve different G proteins. The μ receptor is essential in the activation via all three routes as the locomotor response is abolished in mice lacking this receptor (Becker et al., 2000; Sora et al., 2001a; Tian et al., 1997). The first two are widely referred to as a dopamine independent and a dopamine dependent mechanism (Kalivas et al., 1983). The dopamine independent pathways engage primarily opioid receptors on neurons in the nucleus accumbens (Hakan and Henriksen, 1989; Herkenham et al., 1984), but may also involve non-dopaminergic neurons in the ventral tegmental area that project to the accumbens (Hakan and Henriksen, 1989). Stimulation of accumbens opioid receptors increases locomotor activity in rodents even in the

presence of the mixed D1/D2 receptor antagonist, fluphenazine or after the destruction of dopaminergic terminals by 6-hydroxydopamine (Kalivas et al., 1983). The dopamine dependent pathway relies on dopaminergic neurons in the ventral tegmental area. Under normal conditions, these neurons are inhibited by their GABAergic neighbours tonically. However, when morphine or other opioids are present, they bind to μ receptors located on the GABAergic neurons, suppressing their activity and causing the inhibition on the dopaminergic cells to be relieved (Johnson and North, 1992; Tanda and Di Chiara, 1998). As a result, the dopaminergic neurons become more active, and release more dopamine from their synaptic terminals in the nucleus accumbens (Barrot et al., 1999; Cadoni and Di Chiara, 1999; Tanda and Di Chiara, 1998), leading to the motor hyperactivity observed in the animals (Kalivas and Duffy, 1990).

The third mechanism involves corticosterone, which has been found to be capable of elevating dopamine level in the shell of the nucleus accumbens (Barrot et al., 2000) through the glucocorticoid receptor (Marinelli et al., 1998). Since morphine stimulates the hypothalamic pituitary axis (Buckingham and Cooper, 1986) to induce secretion of corticosterone through the μ receptor (Roy et al., 2001), this morphine elicited rise in corticosterone level may represent another mechanism whereby morphine can produce an increase in locomotor activity. The essential contribution of corticosterone in morphine induced hyperactivity is demonstrated by the significant reduction in motor activation, which is brought about by adrenalectomy (Marinelli et al., 1994) and when glucocorticoid receptors are blocked (Marinelli et al., 1998).

The locomotor response of the $G\alpha_z$ knockout mouse to morphine was examined in this Chapter. The data presented here suggests that G_z plays an important and complex role in the regulation of morphine stimulated motor behaviour.

4.2 Results

4.2.1 Morphine induced locomotor activation is altered in the $G\alpha_z$ knockout mouse.

I began by comparing the locomotor activities of adult $G\alpha_z$ knockout and wildtype mice in response to various doses of morphine. The responses were examined over a time

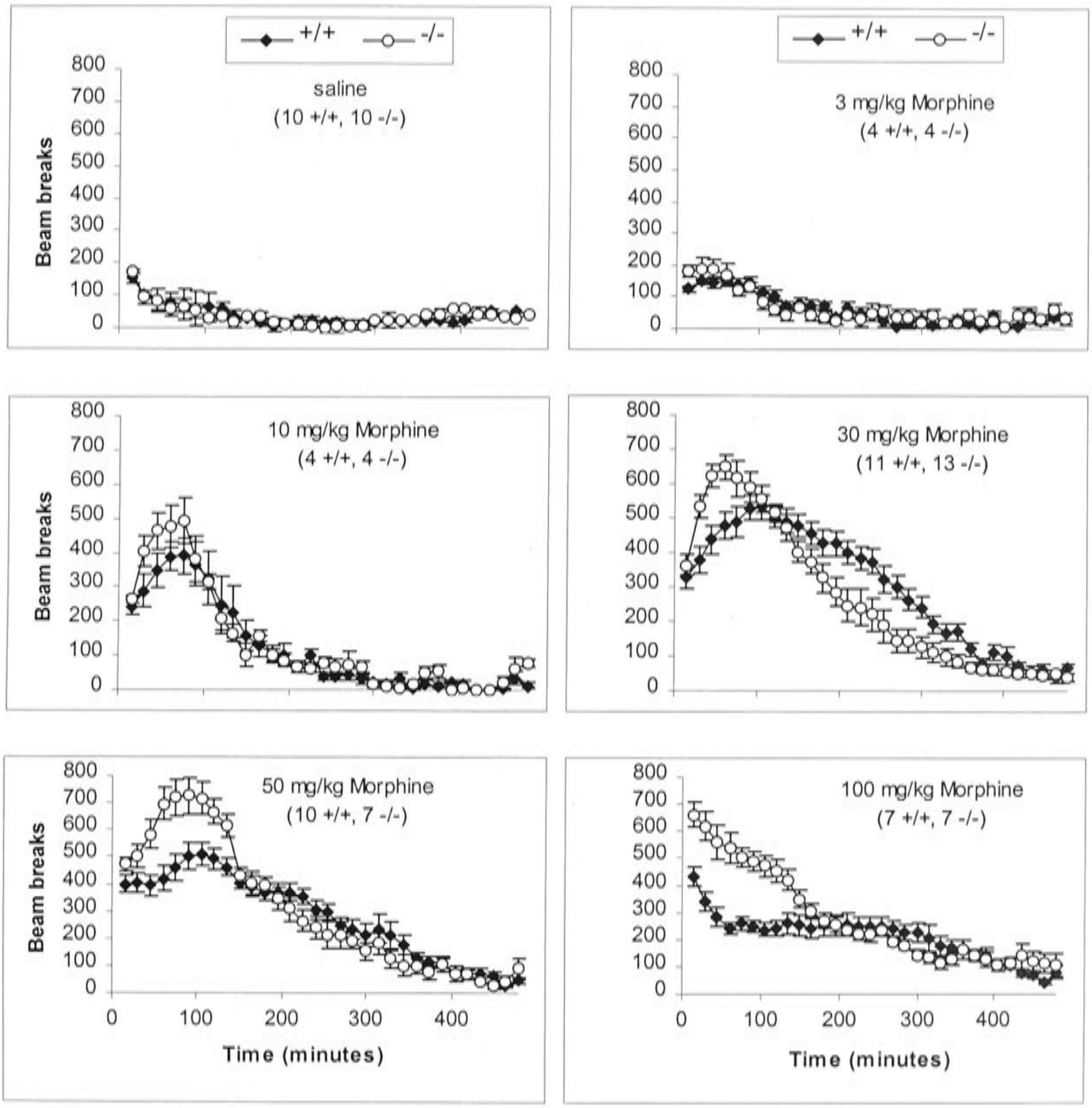


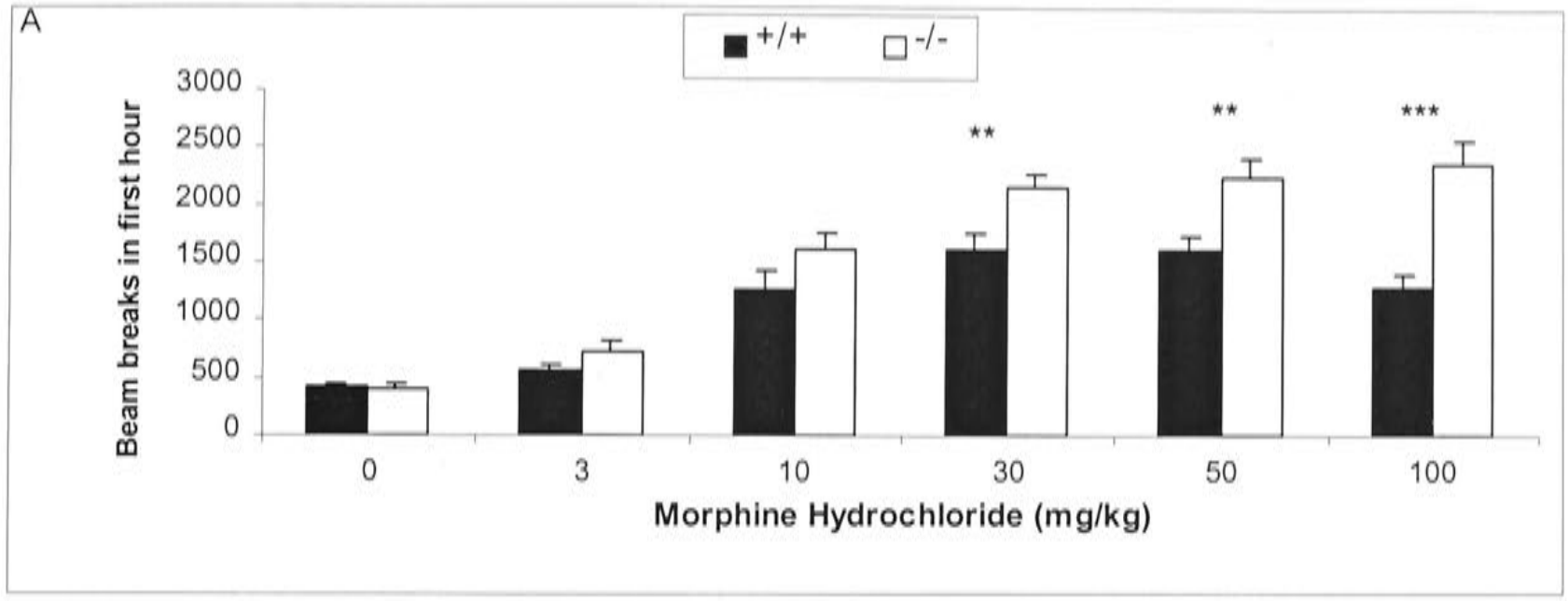
Figure 4.1: Altered morphine induced locomotor activation in the $G\alpha_z$ knockout mouse. Adult wildtype and $G\alpha_z$ knockout mice were treated with either saline vehicle, 3mg/kg, 10mg/kg, 30mg/kg, 50mg/kg or 100mg/kg morphine hydrochloride and locomotor activity of the mice were monitored for 6 hours.

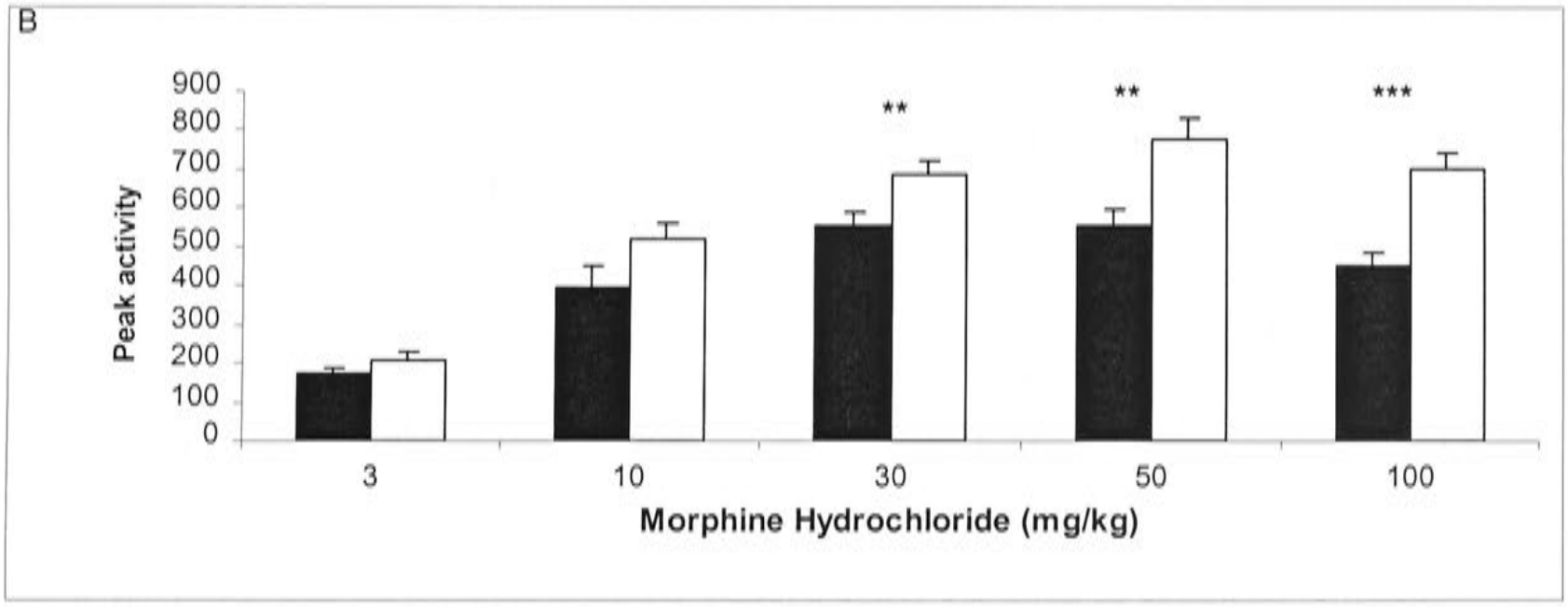
period of six hours in order to capture the full activity profile (Figure 4.1). Overall, relative to wildtype controls, the activity profiles of mice that lack $G\alpha_z$ are characterized by a more rapid rise to a higher peak, and a shortening of the response (Two-way ANOVA: genotype x dose). The initially greater locomotor activation in $G\alpha_z$ knockout mice is reflected by the total activity measure in the first hour after morphine injection, which showed a highly significant genotype main effect ($F(1,61)= 28.1$, $p<.001$) (Figure 4.2a). Peak locomotor activity also demonstrated a very significant genotype main effect ($F(1,61)= 27.6$, $p<.001$), indicating the maximum locomotor stimulation inducible by morphine is greater in $G\alpha_z$ knockout mice (Figure 4.2b). The duration of response analysis also showed a highly significant genotype main effect ($F(1,61)= 15.6$, $p<.001$), suggesting that the duration of the morphine response has been shortened as a consequence of $G\alpha_z$'s absence (Figure 4.2c). The genotype by dose interactions of all three analyses did not reach statistical significance, indicating that the genotype main effect did not depend on dose. This concurred with the consistent trend for $G\alpha_z$ knockout mice to exhibit greater motor activity during the first hour following morphine injection, higher peak activity and a shorter response duration across all doses tested.

Since morphine stimulated motor behaviour undergoes changes during the adolescent period (postnatal day 33-43) (Spear et al., 1982) due to brain maturation (Moll et al., 2000), I investigated whether pre-adolescent mice (postnatal day 24-27) would have a similar response profile to morphine. When pre-adolescent $G\alpha_z$ knockout mice were injected with 50mg/kg of morphine, their locomotion was characterized by an instantaneous rise to the peak and a shortened duration of response ($t(12) = 2.55$, $p<.05$) (Figure 4.3). Locomotor activity in the first hour following morphine treatment was also significantly enhanced in adolescent $G\alpha_z$ knockout mice ($t(12)= 2.92$, $p<.05$). Therefore, the alteration in morphine stimulated motor behaviour as a result of the absence of $G\alpha_z$ developed early in life.

4.2.2 The enhanced locomotor activation to morphine cannot be explained by an increase in plasma corticosterone levels

The initial climb to the peak in motor activity after morphine administration is likely to be contributed by a number of factors. One of them may be the plasma level of corticosterone, which exerts a profound influence on morphine stimulated motor





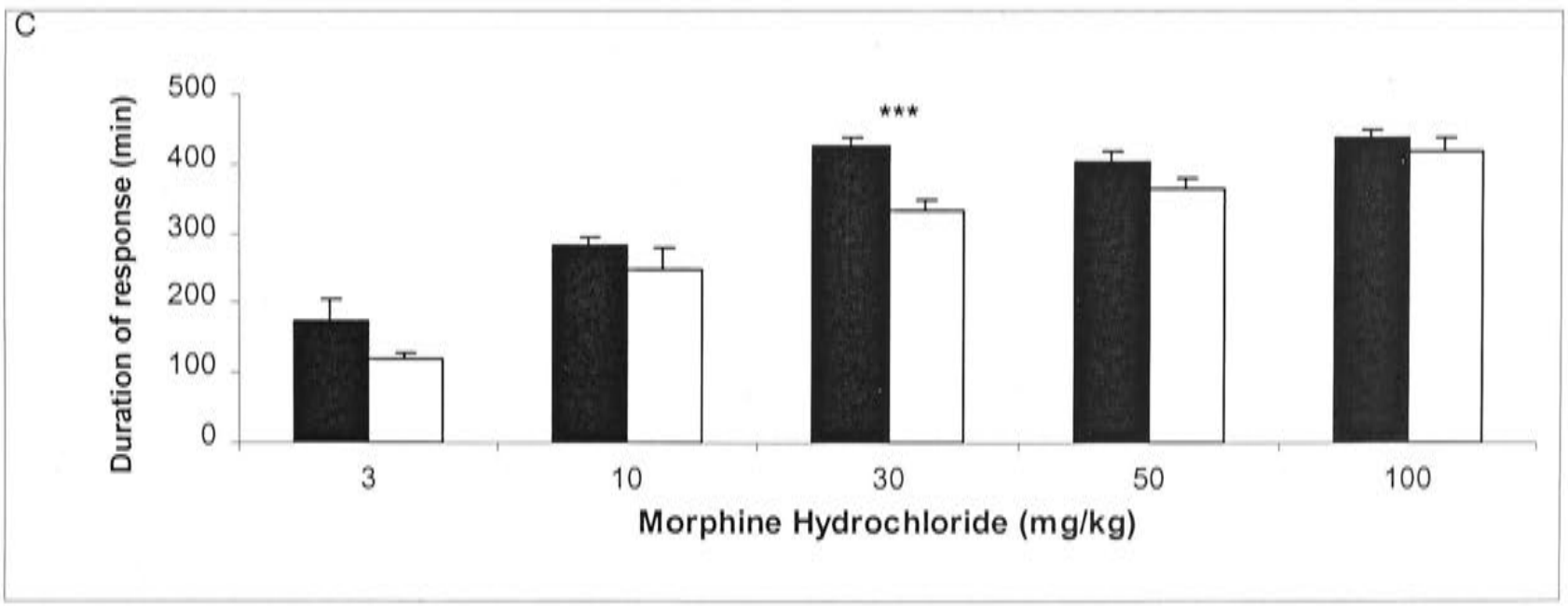
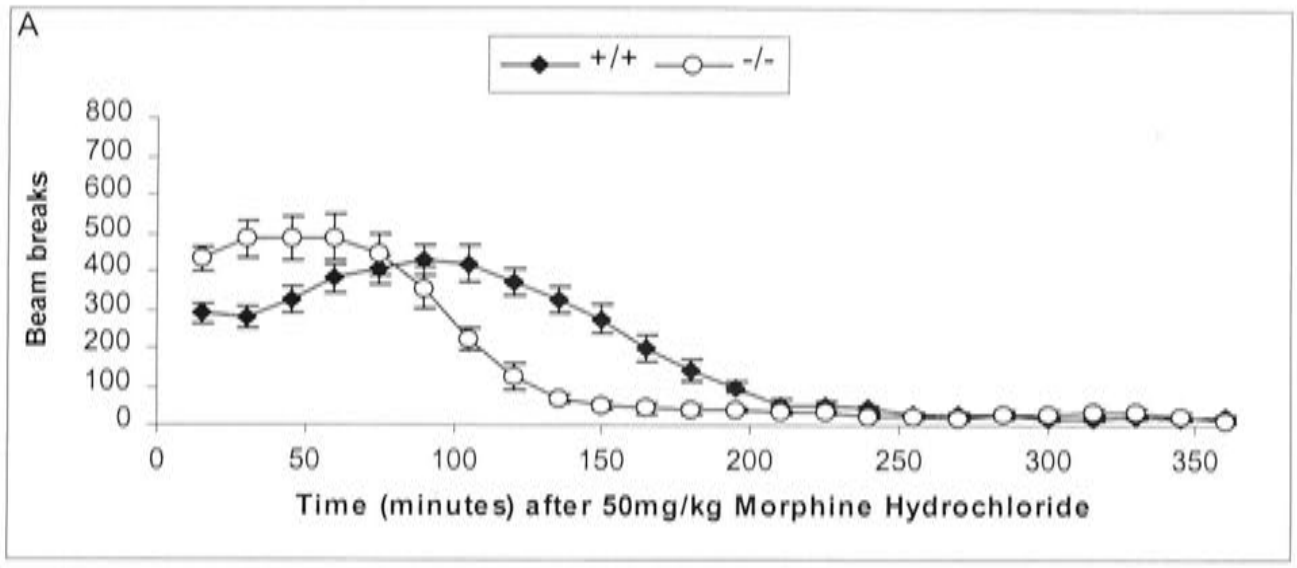
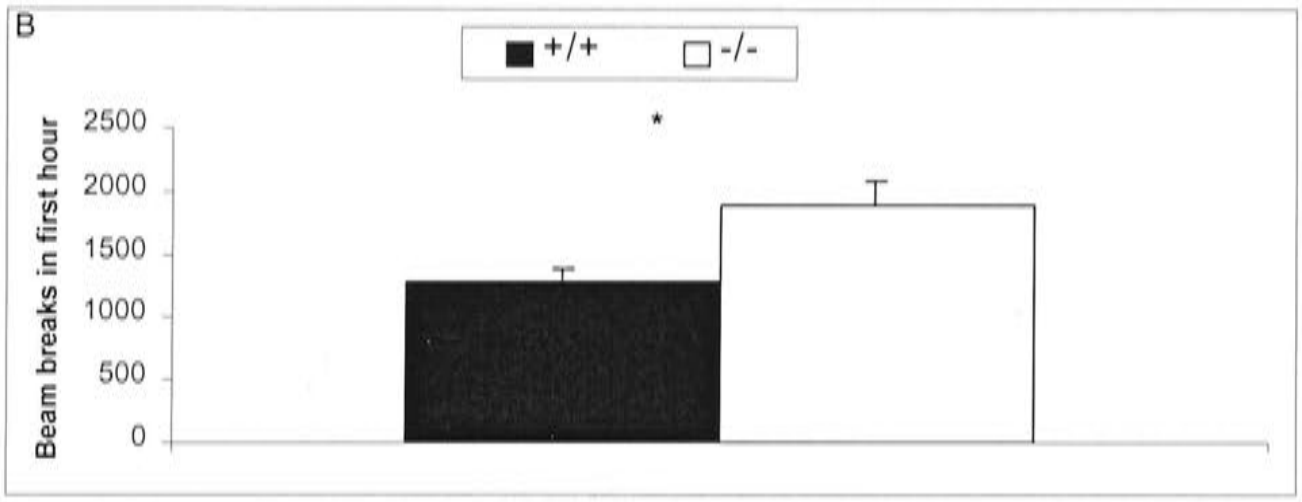
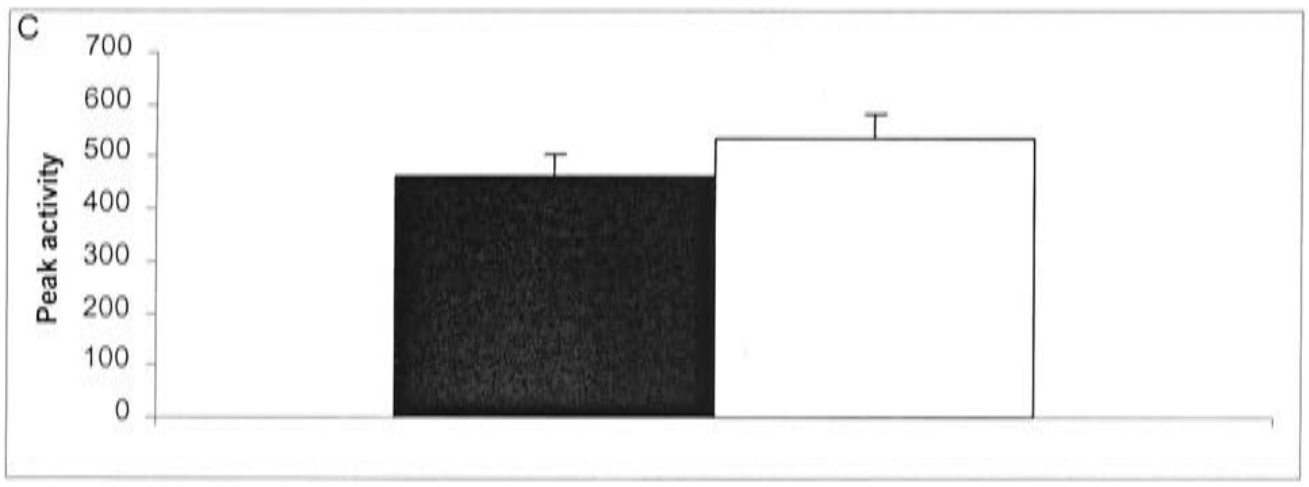


Figure 4.2: Analyses of morphine induced locomotor activation in the $G\alpha_z$ knockout mouse. (A) Total activity (beam breaks) in the first hour following morphine injection. (B) Peak activity (C) Duration of the morphine induced response. The statistical analyses here were performed using the student's t test +/+ vs -/-: *p<.05, ** p<.01, ***p<.001. Due to the small number of animals at some of the doses assessed (see Figure 4.1), there was insufficient power to detect a significant difference using this statistical tool. However, the significant trend due to the effect of genotype across all doses, result in a significant main effect for genotype, and no interaction with dose in the overall ANOVA (see Results).







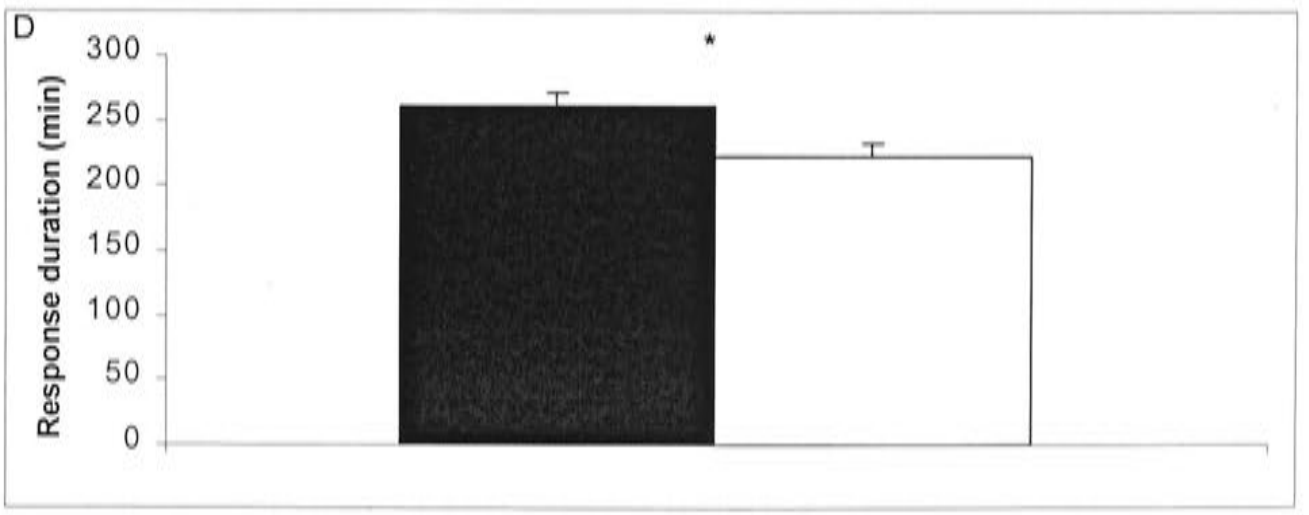


Figure 4.3: Altered morphine induced locomotor activation in adolescent wildtype and $G\alpha_z$ knockout mice (8 +/+, 6-/-). (A) Time course. (B) Total activity in the first hour following morphine administration. (C) Peak activity. (D) Duration of the response. Student's t test +/+ vs -/-: *$p<.05$.

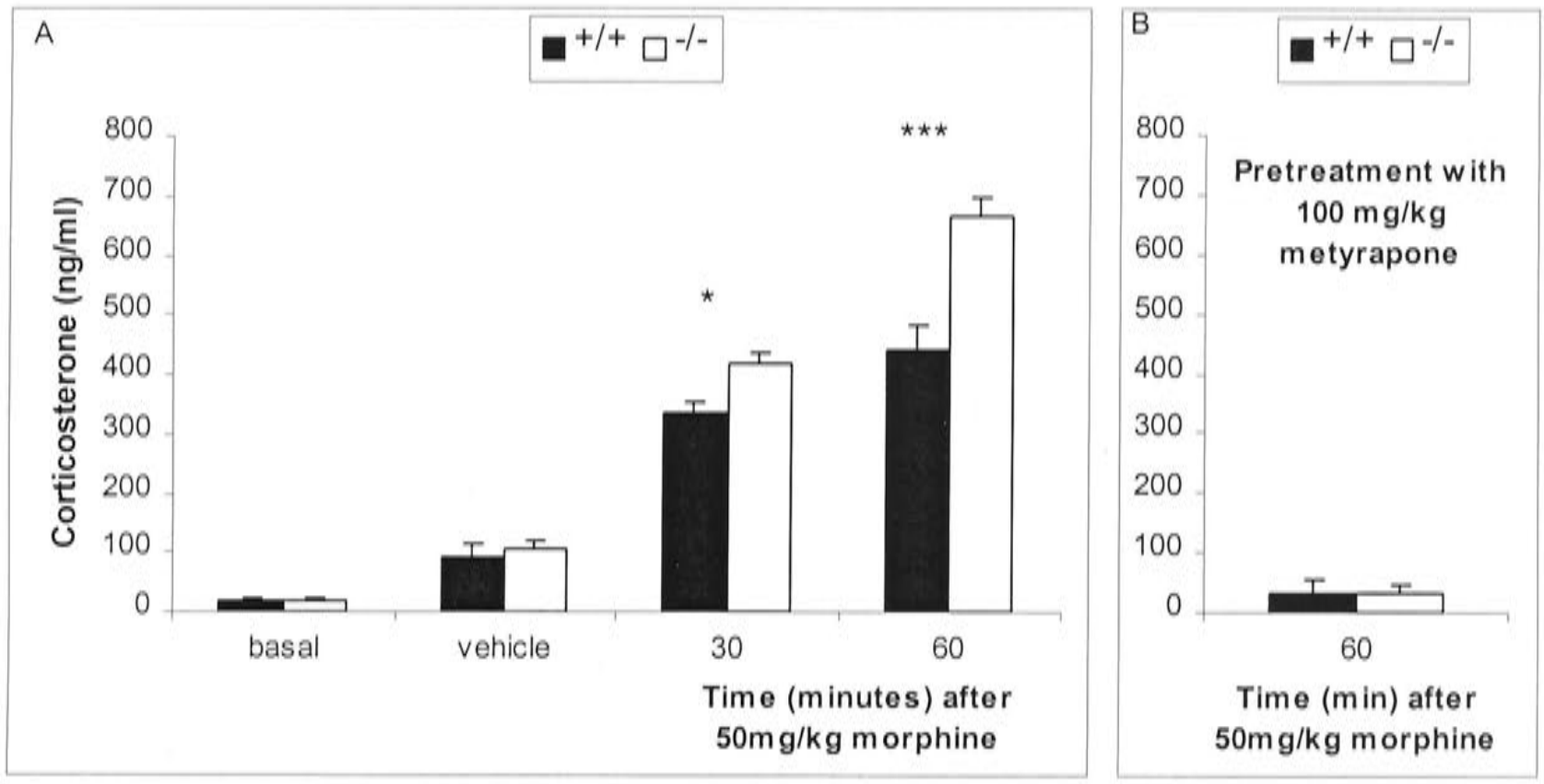
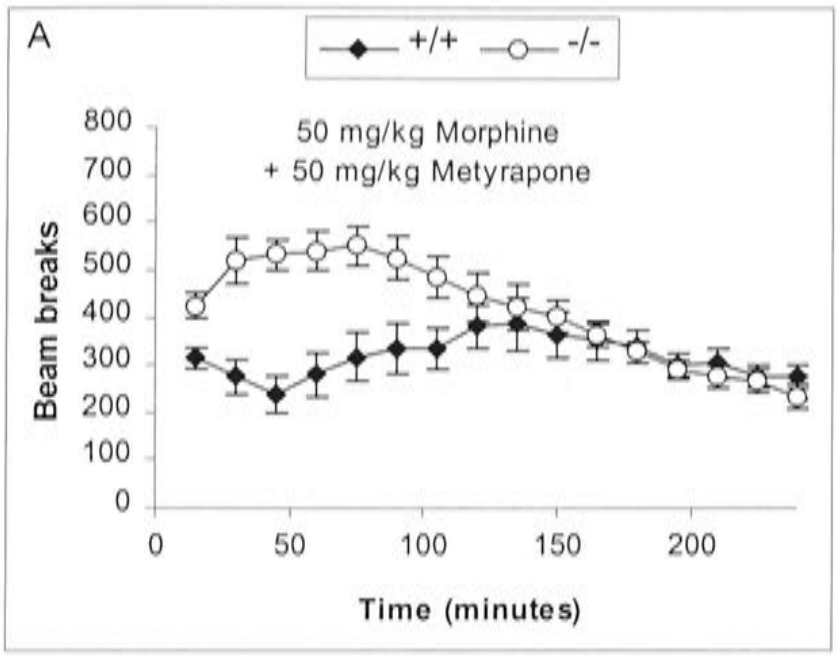


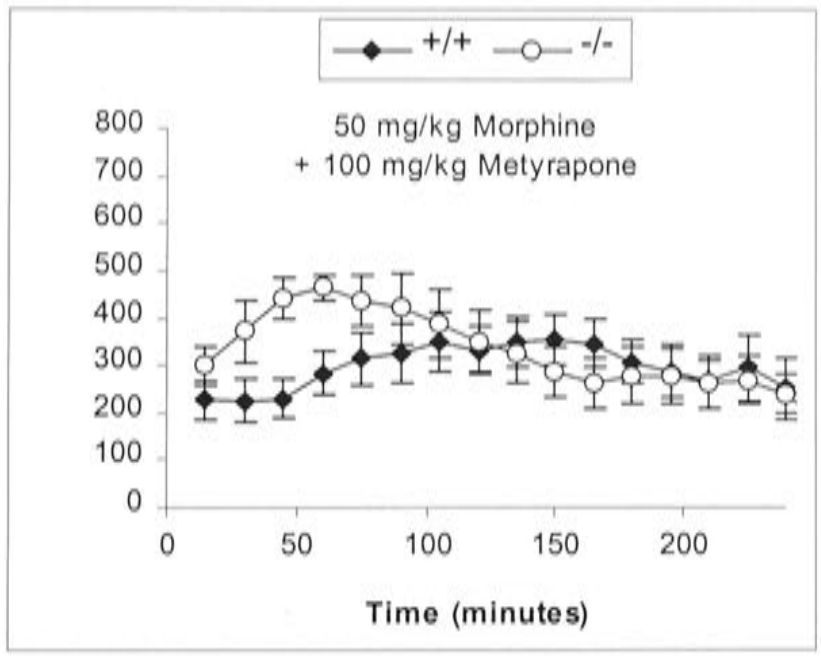
Figure 4.4: Morphine induced significantly greater corticosterone secretion in the $G\alpha_z$ knockout mouse. (A) Corticosterone levels were determined in mice that were not treated (14 +/+, 19 -/-), mice treated with saline vehicle (10 +/+, 12 -/-); and mice treated with 50mg/kg morphine and bled 30 minutes (8 +/+, 8 -/-) or 60 minutes (7 +/+, 7 -/-) after the injection. (B) Pretreatment of mice (7 +/+, 7 -/-) 2 hours beforehand with 100mg/kg metyrapone abolished the corticosterone rise induced by morphine.

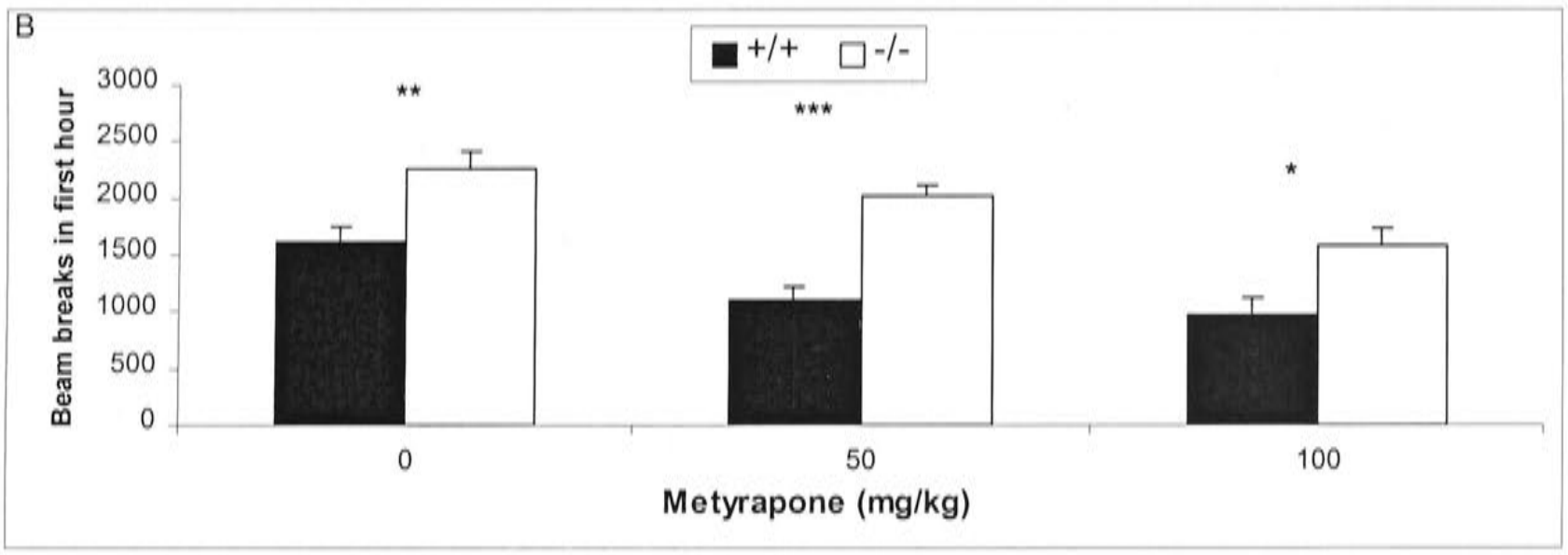
behaviour (Deroche et al., 1995; Frances et al., 2000; Marinelli et al., 1994; Stohr et al., 1999). I measured the levels of plasma corticosterone in wildtype and $G\alpha_z$ knockout mice after morphine administration and found the levels to be significantly augmented in the mutant mice (Figure 4.4a). Therefore, I proceeded to investigate whether this higher plasma corticosterone level is responsible for the greater initial locomotor activation in the $G\alpha_z$ knockout mouse. The corticosterone synthesis inhibitor, metyrapone, was used to prevent the elevation of corticosterone produced by morphine in mice of both genotypes (Figure 4.4b). Consistent with a role for corticosterone in morphine stimulated motor behaviour (Two-way ANOVA: genotype x dose), metyrapone caused a dose dependent reduction in peak activity ($F(2,35)= 8.04$, $p<.002$) and locomotor activity during the first hour after morphine administration ($F(2,35)= 11.8$, $p<.001$) in mice of both genotypes (Figure 4.5). However, the $G\alpha_z$ knockout mouse still demonstrated greater locomotor activation ($F(1,35)=38.5$, $p<.001$: significant genotype main effect for total activity during the one hour after morphine treatment and $F(1,35)= 20.6$, $p<.001$: significant genotype main effect for peak activity, the interaction terms in both analyses were not significant). Therefore, there are other more pertinent factors that contribute to the enhanced locomotor stimulation by morphine in the $G\alpha_z$ knockout mouse.

4.2.3 Examination of κ opioid receptor mediated inhibition of locomotor activity in the $G\alpha_z$ knockout mouse.

Although morphine stimulated locomotor activity has been attributed to the μ receptor at morphine doses ≤ 10mg/kg (Becker et al., 2000; Sora et al., 2001a; Tian et al., 1997), it is possible that at the higher morphine doses employed in the present study, morphine which has lower affinities for the δ and κ receptors (Goldstein and Naidu, 1989; Raynor et al., 1994), also activated these receptors (Narita et al., 1993). Since stimulation of the δ receptor increases locomotion (Negri et al., 1999) while stimulation of the κ receptor depresses motor activity (Simonin et al., 1998), the locomotor response profile elicited by high morphine doses might be the sum of the stimulatory and inhibitory influences caused by the activation of all three opioid receptor subtypes. Accordingly, I surmised that if the κ receptor mediated locomotor suppression is transduced through G_z, then the absence of G_z signaling in the $G\alpha_z$ knockout mouse might explain their greater motor activity through removal of a source of inhibitory influence.







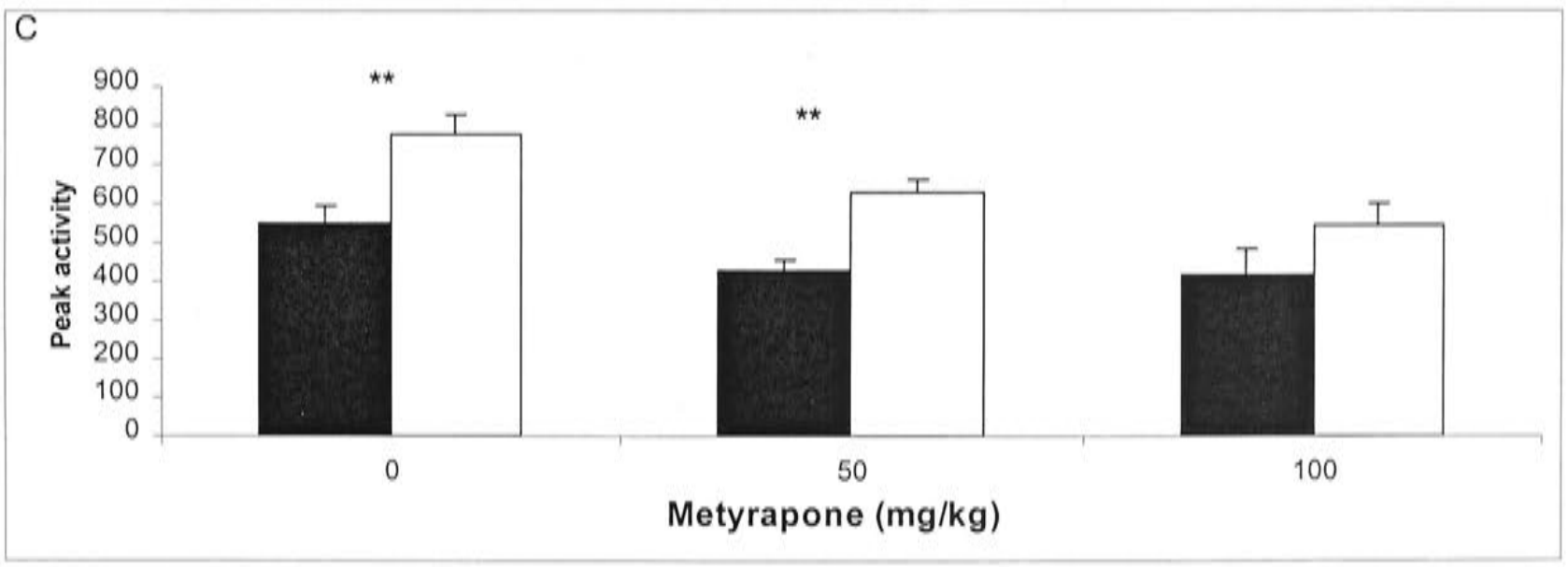


Figure 4.5: The corticosterone synthesis inhibitor, metyrapone reduces locomotor activity in mice. Wildtype and $G\alpha_z$ knockout mice were either pre-treated with 50mg/kg (6 +/+, 6 -/-) or 100 mg/kg (6 +/+, 6 -/-) metyrapone, followed by 50mg/kg morphine hydrochloride 120 minutes later. (A) Locomotor activity profile monitored over 240 minutes after morphine injection. (B) Locomotor activity of mice in the first 60 minutes after morphine injection. (C) Maximum activity inducible by morphine within any 15 minute time bin. The data of mice (10 +/+, 7 -/-) not pre-treated with metyrapone in Figures 4.5b and 4.5c were taken from Figure 4.2. *$p<.05$, ** $p<.01$, ***$p<.001$

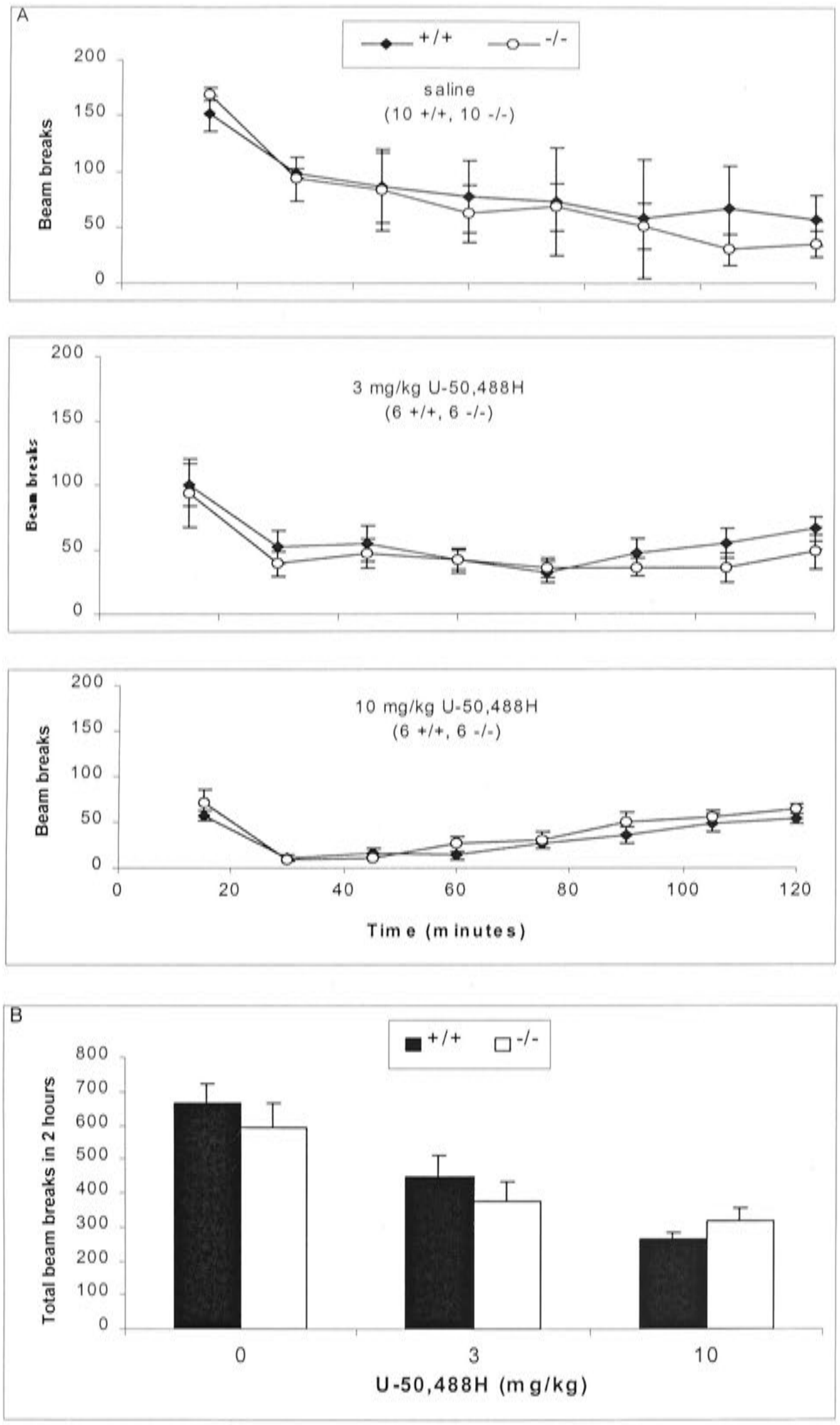


Figure 4.6: Suppression of locomotion by the κ receptor agonist, U-50,488H. Wildtype and $G\alpha_z$ knockout mice were injected subcutaneously with either saline vehicle or 3mg/kg, 10mg/kg U-50,488H. (A) Time course of U-50,488H effects on locomotor activity in wildtype and $G\alpha_z$ knockout mice. (B) Dose dependent depression of locomotor activity in wildtype and $G\alpha_z$ knockout mice by U-50,488H.

To test this hypothesis, I investigated the locomotor responses of wildtype and $G\alpha_z$ knockout mice to the κ receptor agonist, U-50,488H (Two-way ANOVA: genotype x dose). The administration of U-50,488H produced a dose dependent inhibition of locomotor activity (Drug dose main effect: $F(2,38)= 17.0$, $p<.001$) (Figure 4.6). However, there was no difference in the responses of mice from the two genotypes (Genotype main effect: $F(1,38)<1$, NS).

4.2.4 Duration of morphine induced analgesia in the $G\alpha_z$ knockout mouse

Besides stimulating locomotor activity in rodents, morphine is also well known as a powerful analgesic. Since the duration of morphine stimulated locomotor activity appear to be slightly but significantly reduced in $G\alpha_z$ knockout mice (Figures 4.1 to 4.3), I wondered whether the duration of the effects of morphine analgesia would be similarly decreased in these mice. To investigate this possibility, wildtype and mutant mice were administered 50mg/kg of morphine acutely, and morphine analgesia was evaluated using the hotplate test. Intriguingly, relative to wildtype mice, the duration of morphine analgesic effects also appeared to be slightly but significantly reduced in $G\alpha_z$ knockout mice (Genotype main effect: $F(1,21)= 8.99$, $p<.01$) (Figure 4.7).

4.3 Discussion

Mice deficient in $G\alpha_z$ exhibit a complex alteration in morphine stimulated locomotor behaviour, which is characterized by a greater increase in motor activity in the immediate time period following morphine injection and a shortened duration of the morphine response. I have also found morphine to stimulate greater corticosterone secretion in the $G\alpha_z$ knockout mouse. However, this larger rise in plasma corticosterone is likely to play only a secondary role in explaining the increased morphine stimulated motor activity in mutant mice. The augmented locomotor responses of mutant mice were still present even after the differences in morphine induced corticosterone levels have been suppressed by pre-treatment with the corticosterone synthesis inhibitor, metyrapone. I have also shown that there was no difference between mutant and wildtype mice in the inhibition of motor activity caused by activation of the κ receptor. Accordingly, even if high doses of morphine stimulate κ receptors, the greater locomotor activation in $G\alpha_z$ knockout mice is not due to an impairment of κ receptor function.

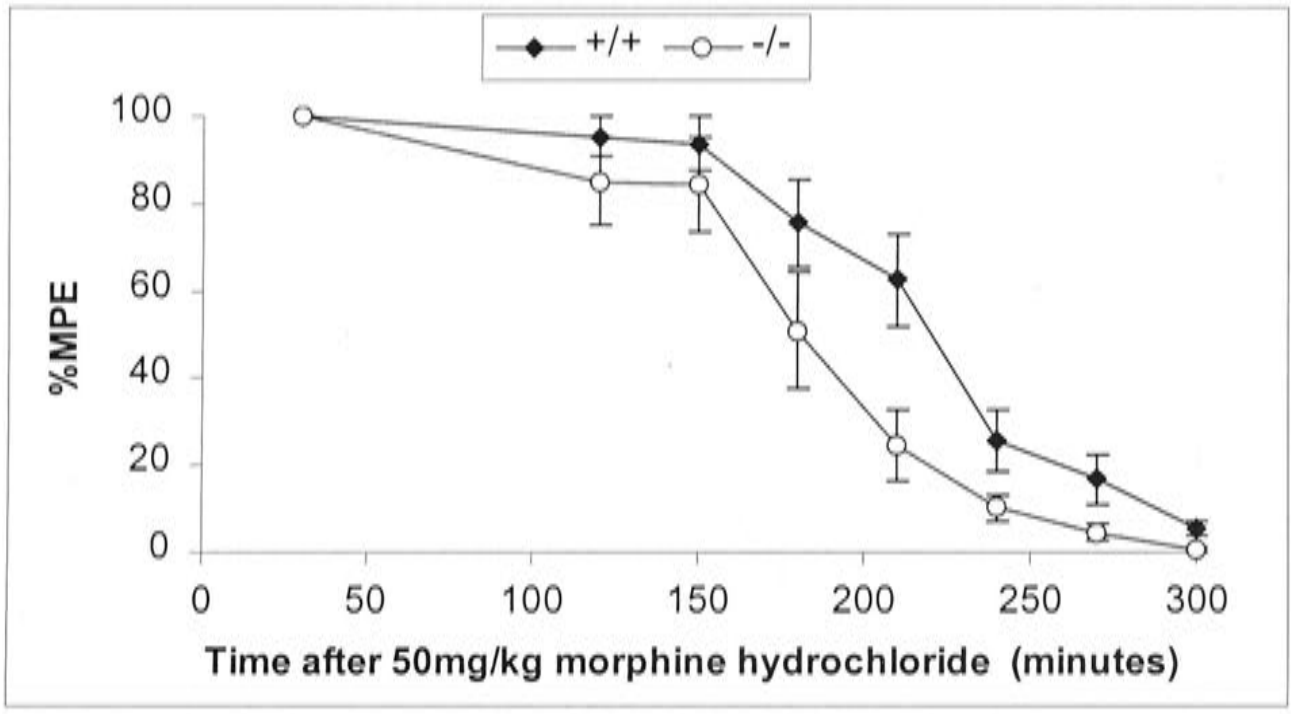


Figure 4.7: The duration of morphine analgesia is reduced in $G\alpha_z$ knockout mice. Wildtype and $G\alpha_z$ knockout mice (12 +/+, 11 -/-) were injected subcutaneously with 50mg/kg morphine hydrochloride and morphine analgesia was evaluated using the hotplate test at 30 minutes, at 120 minutes, and then every half hour thereafter.

Despite the purported involvement of dopamine independent pathways in the locomotor activity stimulated by morphine (Kalivas et al., 1983), the importance of dopamine dependent mechanisms is highlighted by the abolition of the response in mice lacking either the dopamine transporter (Spielewoy et al., 2000) or the dopamine D1 receptor (Becker et al., 2001). Coincidentally, cocaine which increases locomotor activity via a dopaminergic mechanism (Uhl et al., 2002), also stimulates greater locomotor activation in $G\alpha_z$ knockout mice (Yang et al., 2000). An alteration of dopaminergic neurotransmission in mutant mice might therefore account for their augmented response to both morphine and cocaine. Although there is currently no evidence that G_z couples to dopamine receptors *in vivo*, *in vitro* experiments have shown G_z to be capable of coupling to dopamine D2, D3, D4 (Obadiah et al., 1999) and D5 receptors (Sidhu et al., 1998). Morphine and cocaine have been reported to induce a greater elevation of extracellular dopamine concentration in the striatum when presynaptic dopamine D2 autoreceptors are absent (Rouge-Pont et al., 2002). Since dopamine D2 and D3 receptors are present in the nucleus accumbens (Bancroft et al., 1998; Le Moine and Bloch, 1996; Levant, 1998) where some may serve as autoreceptors (Cragg and Greenfield, 1997), and an elevated extracellular dopamine level in the accumbens is related to higher locomotor activity in mice (Kalivas and Duffy, 1990), an impairment of dopamine autoreceptor function in $G\alpha_z$ knockout mice, would help explain their greater locomotor activation by both morphine and cocaine.

The duration of morphine induced locomotor and analgesic effects also appears to be shortened in $G\alpha_z$ knockout mice. The two effects exhibit different time courses (compare Figures 4.1 and 4.7) and are mediated by opioid receptors (of predominantly the μ subtype) in different parts of the brain (Pavlovic and Bodnar, 1998; Tanda and Di Chiara, 1998). The coincidental shortening in the duration of both responses is therefore intriguing and may reflect a unique sequel following the loss of $G\alpha_z$ signaling. Evidence currently available suggests that the μ receptor signals through both G_z and G_{i2} in the brain (Garzon et al., 1997a; Garzon et al., 1997b; Garzon et al., 1998). In the absence of G_z, μ receptor signaling can still occur via G_{i2}. However, compared to $G\alpha_{i2}$ and other G protein α subunits, $G\alpha_z$ is unusual in possessing a very sluggish rate of GTP hydrolysis (Casey et al., 1990). This unique biochemical property allows the activated form of $G\alpha_z$ to remain in the active state for a relatively long period of time until it encounters a $G\alpha_z$ specific GTPase activating protein (Wang et al., 1997). The

data presented here is consistent with the shortened duration of the morphine responses being related to the loss of μ receptor mediated $G\alpha_z$ signaling.

Another notable finding in the present study is the greater stimulation of corticosterone release by morphine in $G\alpha_z$ knockout mice. The mechanism by which morphine elevates plasma corticosterone levels is not well understood and could be mediated indirectly through the effects of morphine on other neurotransmitter systems (Pechnick, 1993). However, the μ receptor is clearly essential for this effect as it was abolished in mice lacking the receptor (Roy et al., 2001). On the other hand, two recently discovered opioid peptides, endomorphin-1 and endomorphin-2, which bind with high specificity and affinity to the μ receptor (Zadina et al., 1997), failed to stimulate plasma corticosterone levels when injected intracerebroventricularly (Coventry et al., 2001). This led the authors to propose that morphine may activate a different subset of μ receptors compared to the endomorphins (Coventry et al., 2001). Notwithstanding, the current result cannot be explained by a decrease in μ receptor signaling as a result of the loss of $G\alpha_z$. The data suggests indirectly that the morphine stimulated corticosterone response must involve at least one other G_z coupled receptor, which normally inhibits corticosterone secretion.

In conclusion, I have shown that G_z is involved in the regulation of morphine stimulated locomotor responses. The locomotor activating effects of psychostimulants are often related to their rewarding effects (Wise, 1987). G_z may therefore play a role in determining the vulnerability of drug users to become dependent on addictive drugs.

Chapter 5

G_z couples to dopamine D2-like receptors *in vivo.*

5.1 Introduction

Dopamine is involved in the regulation of many behavioural and physiological functions within the body, including motor processes (Gerfen, 1992; Jackson et al., 1989), learning (Graybiel, 1995), motivated behaviour (Kelley and Berridge, 2002), body temperature regulation (Boulay et al., 1999a; Lipton and Clark, 1986) and pituitary hormone release (Borowsky and Kuhn, 1992; Diaz-Torga et al., 2002; Durham et al., 1998). Dopaminergic pathways also participate in the locomotor stimulating and rewarding effects of addictive drugs (De Vries and Shippenberg, 2002; Everitt and Wolf, 2002; Herz, 1998; Spanagel and Weiss, 1999). The effects of dopamine on nervous tissues are exerted through cell surface receptors coupled to G proteins. To date, a total of five dopamine receptors have been cloned in mammals, and they have been classified to belong to one of two families according to their pharmacological profiles, amino acid sequence homology in their hydrophobic domains and the structure of their genes (Missale et al., 1998). The dopamine D1-like receptor family consists of dopamine D1 and D5 receptors. They are coupled to G proteins from the G_s family to mediate stimulation of adenylate cyclase (Corvol et al., 2001; Sidhu and Niznik, 2000). Additionally, the D1 receptor may also couple to G_o (Kimura et al., 1995), G_i (Uh et al., 1998) and G_q (Jin et al., 2001) and the D5 receptor may couple to G_z (Sidhu et al., 1998) and G_q (Jin et al., 2001). The dopamine D2-like receptor family comprises the alternatively spliced D2 long and D2 short receptors (Monsma, Jr. et al., 1989), D3 long and D3 short receptors (Fishburn et al., 1993), as well as D4 receptors (Oak et al., 2000). All of these receptors are coupled to members of the inhibitory GTP binding protein family to mediate inhibition of adenylate cyclase (Obadiah et al., 1999; Sidhu and Niznik, 2000). In addition, there is also evidence that the D3 receptor may couple to G_s (Obadiah et al., 1999) and G_q (Newman-Tancredi et al., 1999).

Most of the studies on G protein coupling of dopamine receptors have either been performed in cell lines by transfection of the receptors and G protein α subunits or in tissues using broken membrane preparations. Although these methods are very useful

for preliminary identification of possible G proteins that a receptor can couple to, the results obtained must be confirmed in intact cells from body tissues, where the presence or absence, as well as the levels of expression of various components in the G protein signaling cascade are tightly regulated (Mende et al., 1998; Zelenin et al., 2002) and the existence of cellular compartments may restrict receptors from accessing G proteins in other parts of the cell (Allgeier et al., 1997; Degtiar et al., 1997; Ostrom et al., 2000). Both of these factors are known to play an important role in determining the specificity of G protein signaling in native tissues (Dumont et al., 2002). In addition, the constitution of the βγ subunits that associate with the α subunit can also have a significant impact on the specificity of receptor coupling (Hou et al., 2000; McIntire et al., 2001; Wang et al., 2001). Differences in the βγ subunit composition of G proteins in various cell lines and tissue types may potentially explain the many discrepant findings in the literature. For instance, whereas G_o but not G_q was found to couple to D1 receptors in one study (Kimura et al., 1995), the reverse conclusion was arrived in another study (Jin et al., 2001). Similarly, G_s could couple to the D3 receptor in one study (Obadiah et al., 1999), but not in another (Newman-Tancredi et al., 1999). The use of native tissues may obviate the laborious task of identifying the specific βγ dimer that associate with the transfected α subunit since the observed receptor G protein coupling would be natural.

For members of the dopamine D2-like receptor family, G_o has been proposed to be the predominant G protein that couple to most receptors in the brain, with G_{i1}, G_{i2} and G_{i3} playing a minor role (Jiang et al., 2001). This conclusion is based on the inability of GTP to displace ^{125}I-sulpiride binding in brain sections from the $G\alpha_o$ knockout mouse when dopamine is present. This suggests that in the absence of $G\alpha_o$, almost all sulpiride sensitive dopamine receptors are in the low affinity G protein uncoupled form, which implicates the involvement of $G\alpha_o$ in receptor coupling. In contrast, high affinity dopamine binding is not affected in mice lacking both $G\alpha_{i1}$ and $G\alpha_{i2}$ or when $G\alpha_{i1}$ and $G\alpha_{i3}$ are absent. In the $G\alpha_i$ mutant animals, dopamine retains its ability to catalyse the exchange of GDP for GTP on the receptor bound G protein. In doing so, the receptor converts to the low affinity form due to G protein uncoupling and this is visualized as the rightward displacement of the ^{125}I-sulpiride binding curve (Jiang et al., 2001).

Since sulpiride and dopamine bind to all D2-like receptors with similar affinities (Missale et al., 1998), the result implies that G_o couples to most D2, D3 and D4 receptors in the brain. However, these data do not preclude the coupling of D2-like receptors to other G proteins. G_o is by far the most abundant G protein in the brain (Sternweis and Robishaw, 1984), and the radiobinding technique used is unlikely to possess sufficient sensitivity to detect dopamine receptor coupling attributed to other significantly less abundant G proteins like G_z. Furthermore, the high affinity receptor binding state of some D2-like receptors is known to be critically dependent on subtle changes in assay conditions, such as magnesium concentration (Bancroft et al., 1998).

The data presented in Chapter 4 suggested that the peak locomotor response to morphine was significantly increased in $G\alpha_z$ knockout mice, and the result could not be explained by the higher morphine induced corticosterone secretion in these mice. $G\alpha_z$ knockout mice also showed a similarly enhanced locomotor stimulatory response to the psychostimulant (Yang et al., 2000). Since the augmentation of locomotor activity elicited by cocaine (Uhl et al., 2002; Xu et al., 1994) and to a certain degree, morphine (Barrot et al., 1999; Cadoni and Di Chiara, 1999; Kalivas et al., 1983; Kalivas and Duffy, 1990) was dependent on central dopaminergic neurotransmission, the increased locomotor activity to both drugs in $G\alpha_z$ knockout mice could imply an alteration in the dopaminergic pathways of these mice. The present study was therefore conducted to examine whether dopamine receptor functions were altered in the $G\alpha_z$ knockout mouse.

5.2 Results

5.2.1 Locomotor activation by amphetamine in the $G\alpha_z$ knockout mice

Since mice lacking $G\alpha_z$ have been reported to demonstrate greater cocaine induced locomotor activation (Yang et al., 2000), I began by examining whether amphetamine stimulated locomotor activity are also augmented in these mice. Both amphetamine and cocaine produce an elevation of extracellular dopamine concentrations in the nucleus accumbens, which is believed to contribute to the motor activating effects of these drugs (Pontieri et al., 1995). As shown in Figure 5.1, amphetamine caused a significantly greater locomotor response in $G\alpha_z$ knockout mice (Genotype main effect: $F(1,25)= 17.1$, $p<.001$ compared to wildtype controls). This increase in locomotor activity of $G\alpha_z$ knockout mice occurred throughout most of the duration of the drug effect (Genotype

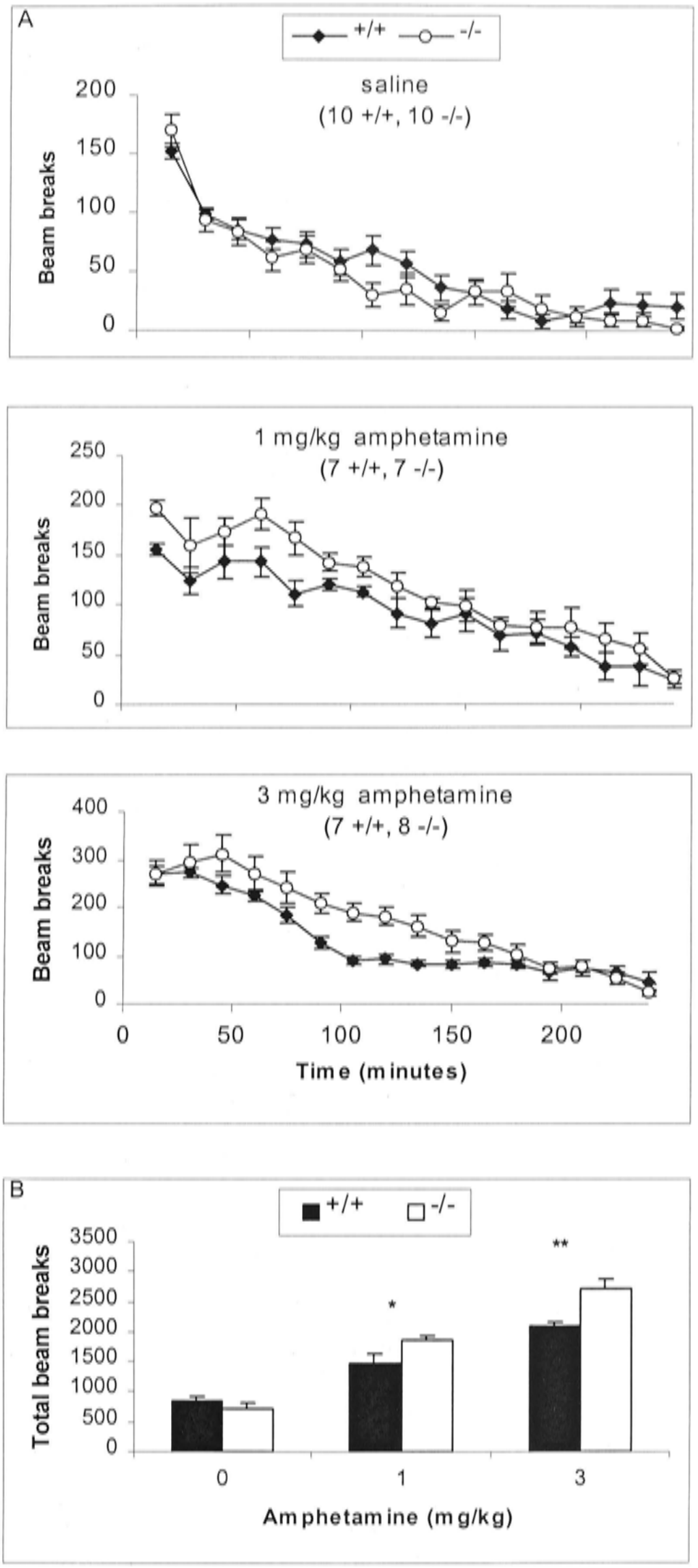


Figure 5.1: Locomotor activation induced by amphetamine is significantly increased in $G\alpha_z$ knockout mice. Mice were given subcutaneous injection of either saline vehicle, 1mg/kg or 3mg/kg *d*-amphetamine and the locomotor activity of the mice was monitored over 4 hours. The values shown are the means ± SEM. (A) Time course and (B) total activity of $G\alpha_z$ knockout and wildtype mice in 4 hours. +/+ vs -/-: *$p<.05$, **$p<.01$

by Time interaction: F(3.9,96.7)=1.8, NS).

5.2.2 The locomotor response of the $G\alpha_z$ knockout mouse to SKF 38393

The motor activating effects of psychostimulants are mediated through dopamine receptors (Ushijima et al., 1995; van den et al., 1988; Xu et al., 1994) (Neisewander et al., 1995; Xu et al., 2000b). The simultaneous activation of postsynaptic D1 and D2-like receptors are believed to be required for stimulation of locomotor activity in rodents (Dreher and Jackson, 1989). Activation of presynaptic D2-like autoreceptors, on the other hand, appear to curb locomotor activity by decreasing dopamine release (Starr and Starr, 1986; Usiello et al., 2000). I tested the locomotor response of the mice to the D1-like receptor agonist, SKF 38393 (Tirelli and Terry, 1993). At 10mg/kg and 100mg/kg, SKF 38393 caused a significant enhancement of locomotor activity in both wildtype and $G\alpha_z$ knockout mice (Figure 5.2) (Drug dose main effect: $F(2,38)= 16.5$, $p<.001$). However, there was no significant difference in the magnitude of locomotor responses between mice of the two genotypes (Genotype main effect: $F(1,38)<1$, NS).

5.2.3 The locomotor response of the $G\alpha_z$ knockout mouse to quinpirole

Since the inhibition of presynaptic D2-like autoreceptors has been shown to increase locomotor activation induced by psychostimulants (Ushijima et al., 1995; van den et al., 1988), I speculated that if the receptor is coupled to G_z, the absence of G_z may lead to an impairment of receptor function, and hence explain the greater locomotor activity in $G\alpha_z$ knockout mice. Therefore, I examined the responses of $G\alpha_z$ knockout mice to low doses of quinpirole, which preferentially act on presynaptic D2-like receptors to inhibit locomotor activity (Usiello et al., 2000; Wang et al., 2000; Zhuang et al., 2001). Consistent with my hypothesis, a significant reduction in the locomotor suppressive effects of quinpirole in $G\alpha_z$ knockout mice was observed (Figure 5.3) (Genotype main effect: $F(1,41)=12.5$, $p<.01$).

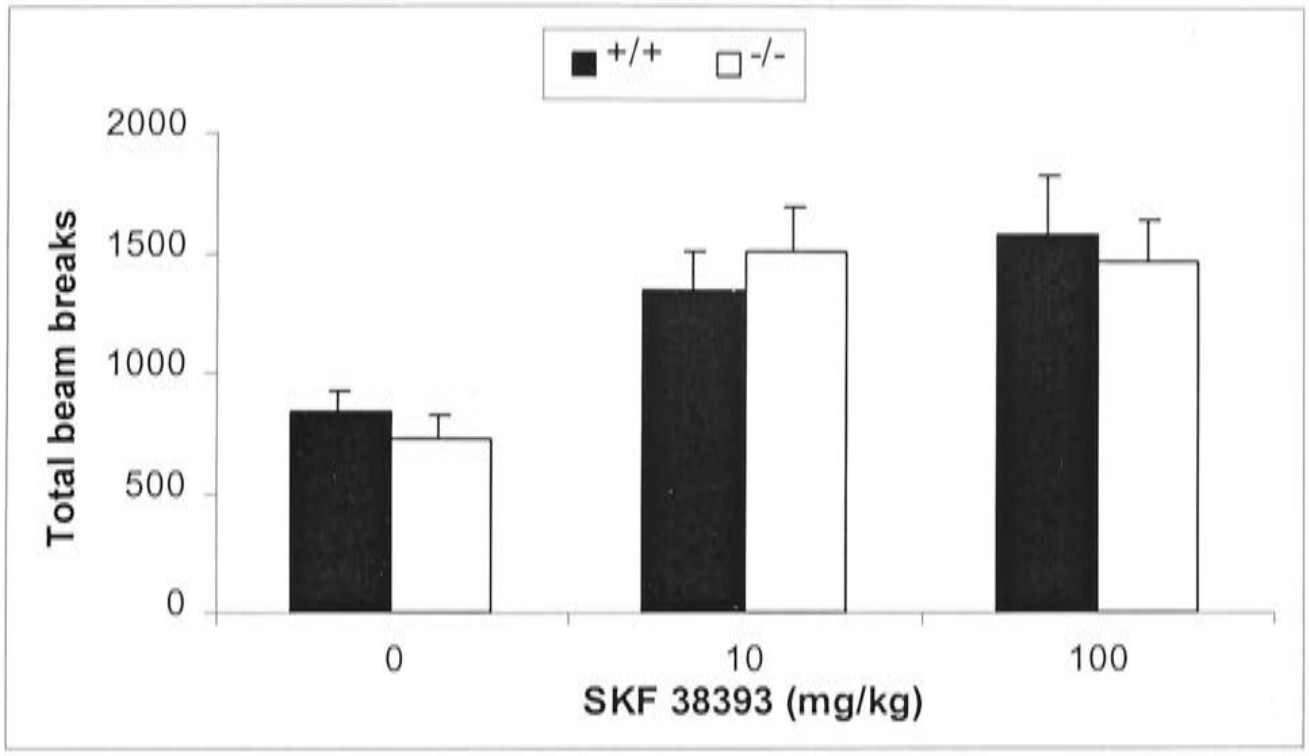


Figure 5.2: Locomotor responses of wildtype and $G\alpha_z$ knockout mice to the D1-like receptor agonist, SKF 38393. Means ± SEM are shown. Mice were injected subcutaneously with saline vehicle (10 +/+, 10 -/-), 10 mg/kg (6 +/+, 6 -/-) or 100 mg/kg (6 +/+, 6 -/-) SKF 38393 and total locomotor activity over 4 hours was measured.

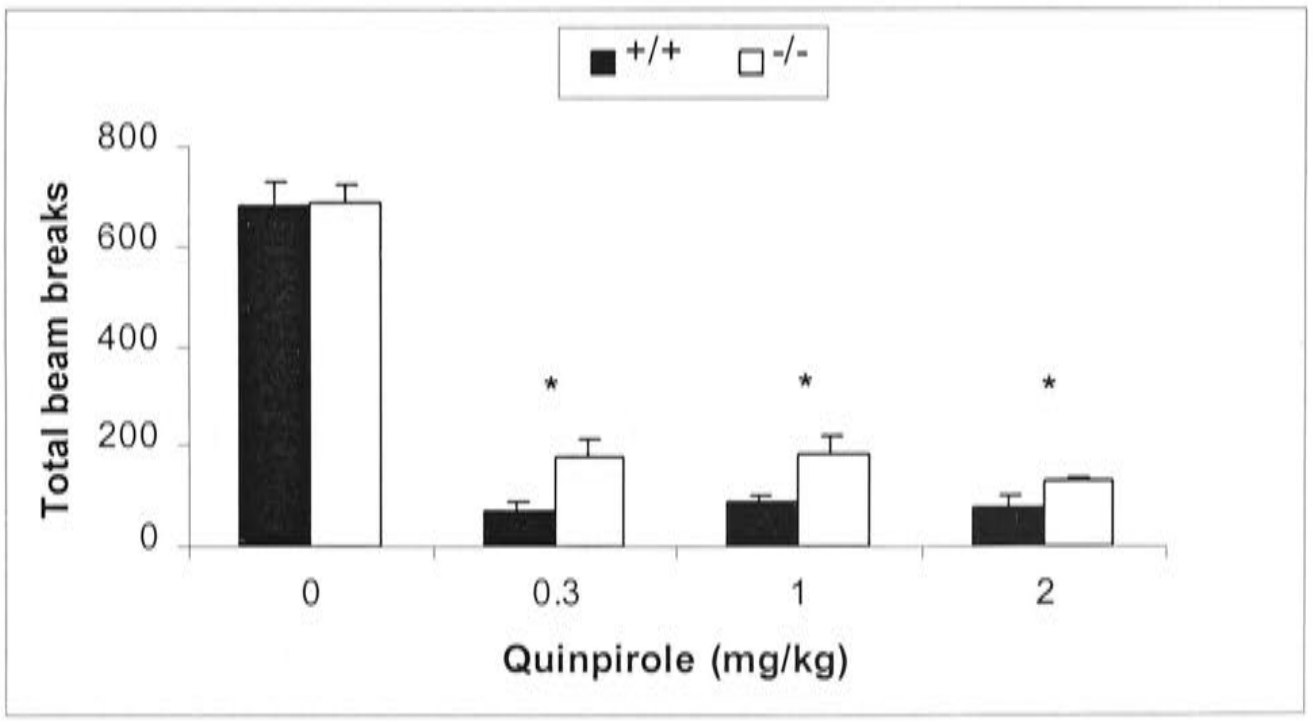


Figure 5.3: Locomotor responses of wildtype and $G\alpha_z$ knockout mice to the D2-like receptor agonist, quinpirole. Means ± SEM are shown. Mice were given a subcutaneous injection of either saline vehicle (10 +/+, 10 -/-), 0.3mg/kg (5 +/+, 7 -/-), 1mg/kg (11 +/+,12 -/-) or 2mg/kg (5 +/+, 7 -/-) quinpirole, and locomotor activity was monitored for 90 minutes. +/+ vs -/-: *$p \leq .05$.

5.2.4 Quinpirole induced inhibition of dopamine release in the nucleus accumbens

Quinpirole has been reported to suppress locomotor activity by acting on dopamine D2-like receptors in the core of the nucleus accumbens (Swanson et al., 1997). To confirm that presynaptic D2-like receptor function has been compromised in the $G\alpha_z$ knockout mouse, the inhibition of electrically evoked dopamine release by quinpirole in the nucleus accumbens core was measured (Figure 5.4). While the infusion of 100ng of quinpirole into the accumbens core inhibited dopamine release by 79.5 ± 1.8% in wildtype mice (n=7) after 15 minutes, the same dose of quinpirole caused only 60.6 ± 4.2% inhibition of dopamine release in $G\alpha_z$ knockout mice (n=8) ($t(13)= 3.88$, $p<.01$) (Figure 5.5). All but one of the mutant mice demonstrated lesser inhibition than wildtype mice 15 minutes after quinpirole infusion. At 30 minutes post-infusion, the difference between the genotypes remained significant ($t(13)= 2.67$, $p<.05$). After 45 minutes, the difference gradually diminished due to data variability caused mainly by a rapid recovery seen in one wildtype mouse. Pre-treatment with 1μg of haloperidol 15 minutes before quinpirole infusion abolished the inhibition of dopamine release by quinpirole (data not shown), suggesting the effect is specific to dopamine D2-like receptors.

5.2.5 Quinpirole induced hypothermia

Stimulation of dopamine D2 receptors also produce hypothermia in mice (Boulay et al., 1999a). There is controversy whether this is mediated via a presynaptic (Zarrindast and Tabatabai, 1992) or postsynaptic (Boulay et al., 1999a; Sanchez and Arnt, 1992) D2 mechanism. I evaluated the hypothermic effects of quinpirole and found the lack of $G\alpha_z$ to significantly attenuate the decrease in body temperature elicited by the drug (Figure 5.6) (Genotype main effect: $F(1,38)= 27.3$, $p<.001$), which occurred at all quinpirole doses tested (Genotype by Dose interaction: $F(2,38)=1.72$, NS).

5.2.6 Quinpirole induced ACTH release

Dopaminergic pathways also participate in the regulation of hormone release. Systemic administration of quinpirole caused a dose-dependent increase in plasma adrenocorticotropic hormone (ACTH) level, which can be antagonized by the D2-like

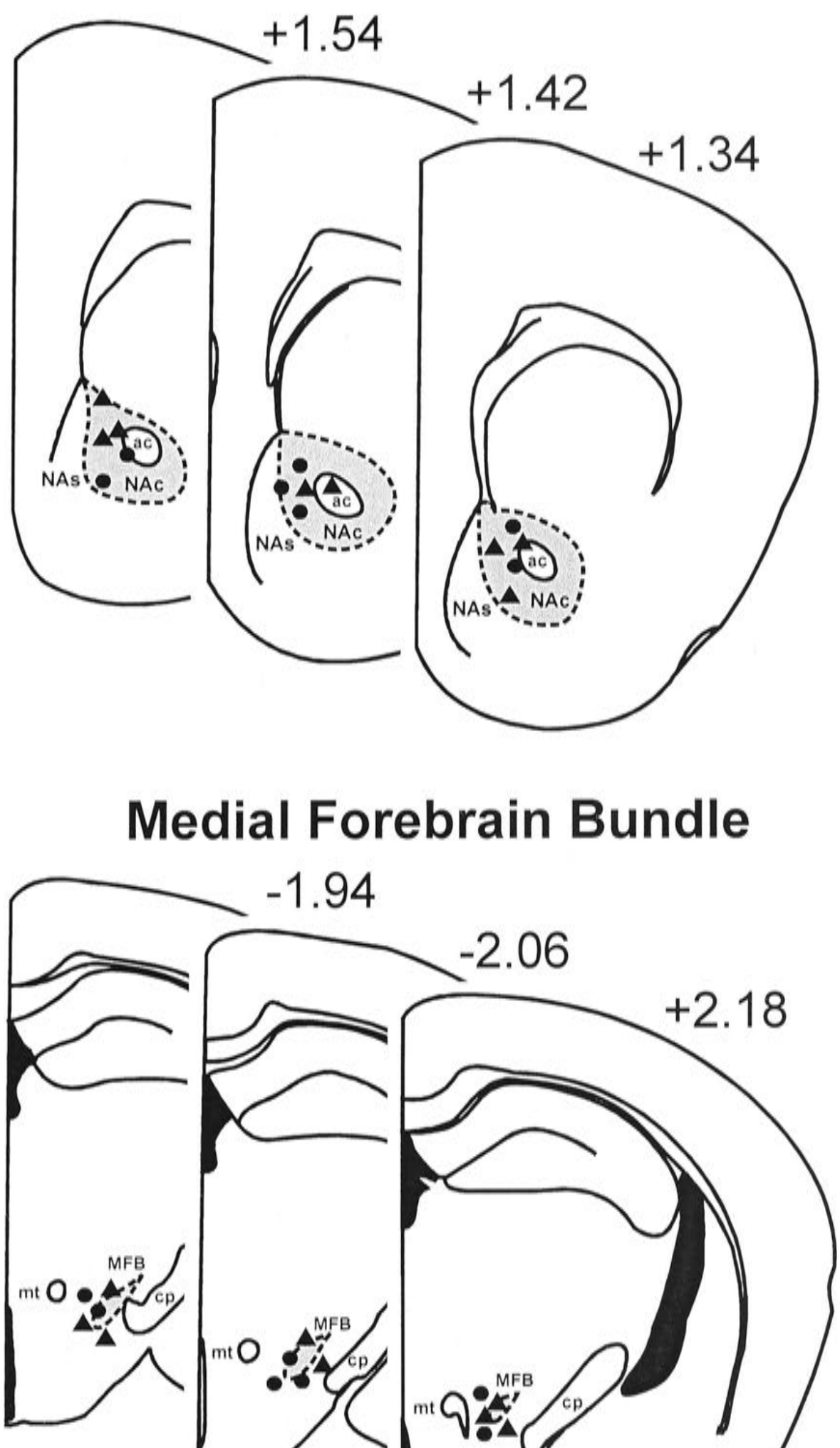


Figure 5.4: Representative coronal sections of the brain showing placement of amperometric recording electrodes in the nucleus accumbens (gray area) and stimulating electrodes in the medial forebrain bundle (gray area) of wildtype (closed circles) and $G\alpha_z$ knockout (closed triangles) mice. The numbers correspond to distance (mm) from Bregma. Abbreviations: NAc, nucleus accumbens core; NAs nucleus accumbens shell; ac, anterior commissure; MFB, medial forebrain bundle; mt, mammillothalamic tract; cp, cerebral peduncle. (The figures are adapted from Franklin and Watson, 1997).

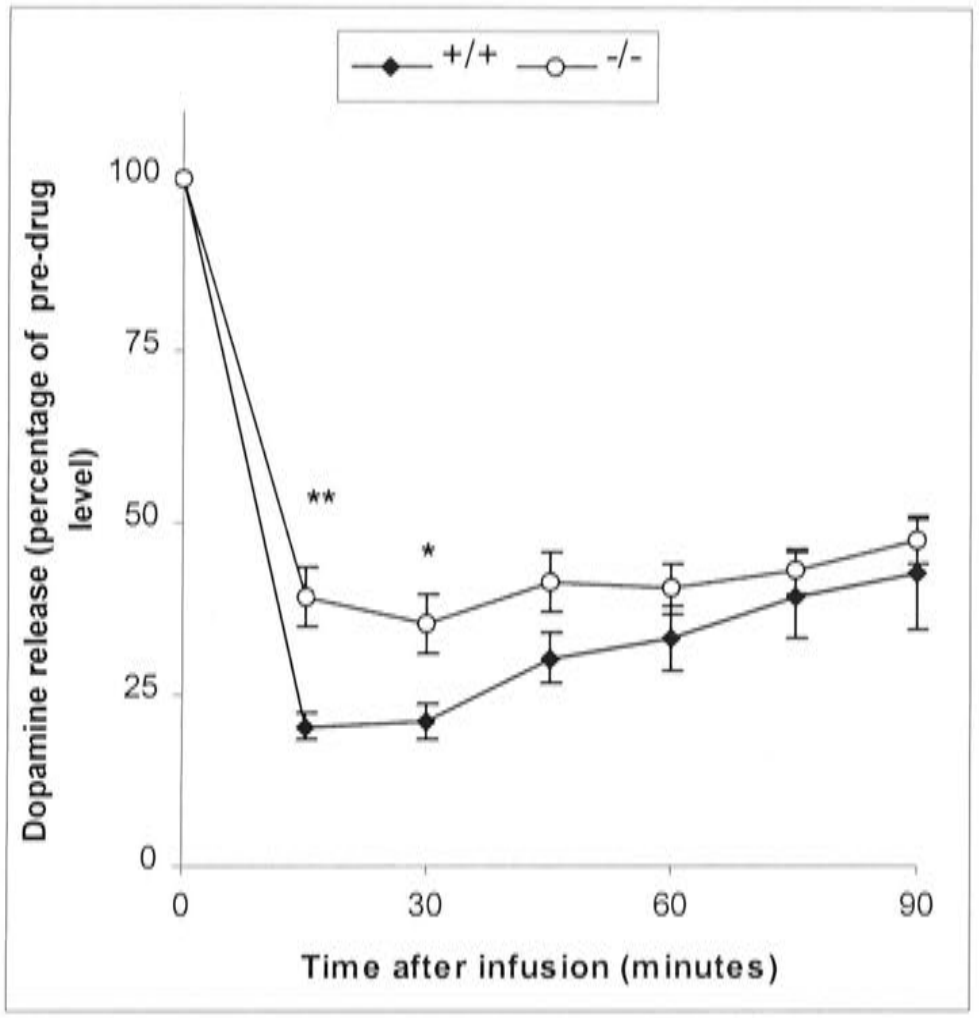


Figure 5.5: Quinpirole inhibition of dopamine release is significantly attenuated in the nucleus accumbens of the $G\alpha_z$ knockout mouse. The release of dopamine from synaptic terminals in the core of the nucleus accumbens was electrically evoked by stimulation of their axons in the medial forebrain bundle. After pre-drug baseline responses have been recorded, 100ng of quinpirole was infused via a cannula into the nucleus accumbens, and dopamine release in the nucleus accumbens core was recorded every 15 minutes for a total duration of 90 minutes. The mean ± SEM responses from 7 +/+ and 8 -/- mice are shown. +/+ vs -/-: *$p<.05$, **$p<.01$.

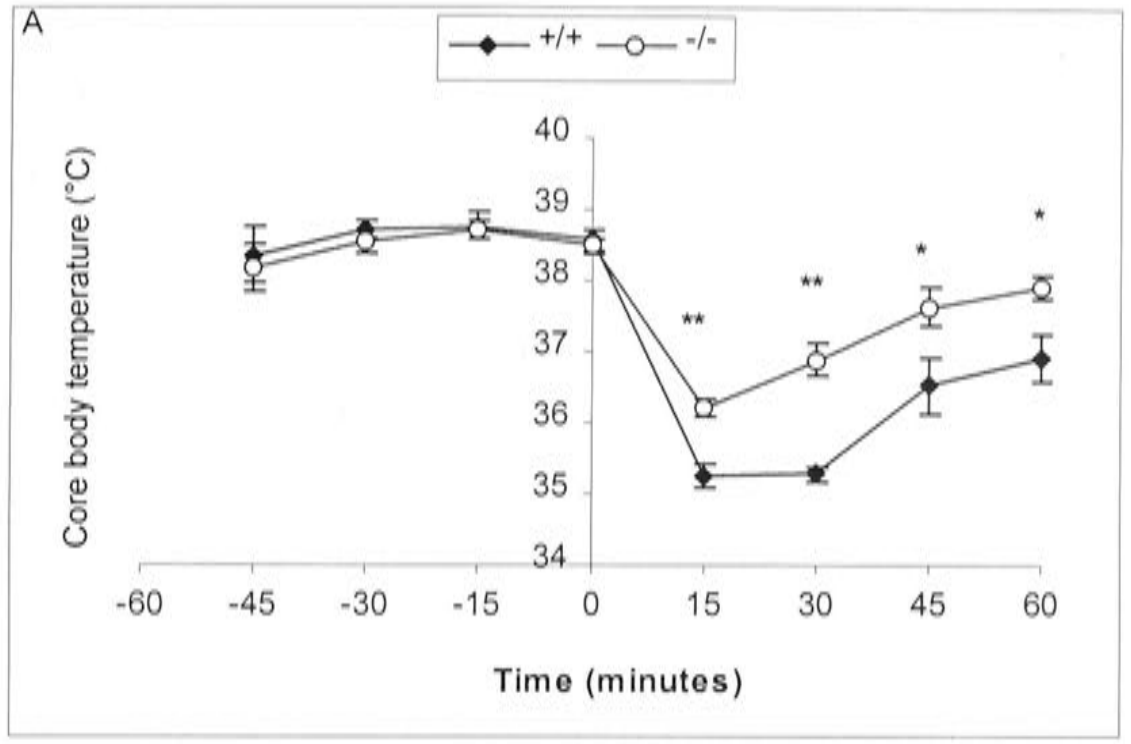


Figure 5.6: Hypothermia induced by quinpirole is significantly reduced in the $G\alpha_z$ knockout mouse. Mice were injected subcutaneously with saline vehicle (7 +/+, 7 -/-), 0.1 mg/kg (8 +/+, 8 -/-), 0.3mg/kg (7 +/+, 7 -/-) or 1 mg/kg (7 +/+, 7 -/-) quinpirole and their body temperatures were measured using a rectal thermistor probe. (A) Change in body temperature of +/+ and -/- mice in response to 1mg/kg quinpirole injected at time 0. The plotted values are the mean body temperatures ± SEM. (B) Hypothermia induced by various doses of quinpirole 15 minutes after injection. +/+ vs -/-: * $p<.05$, ** $p<.01$.

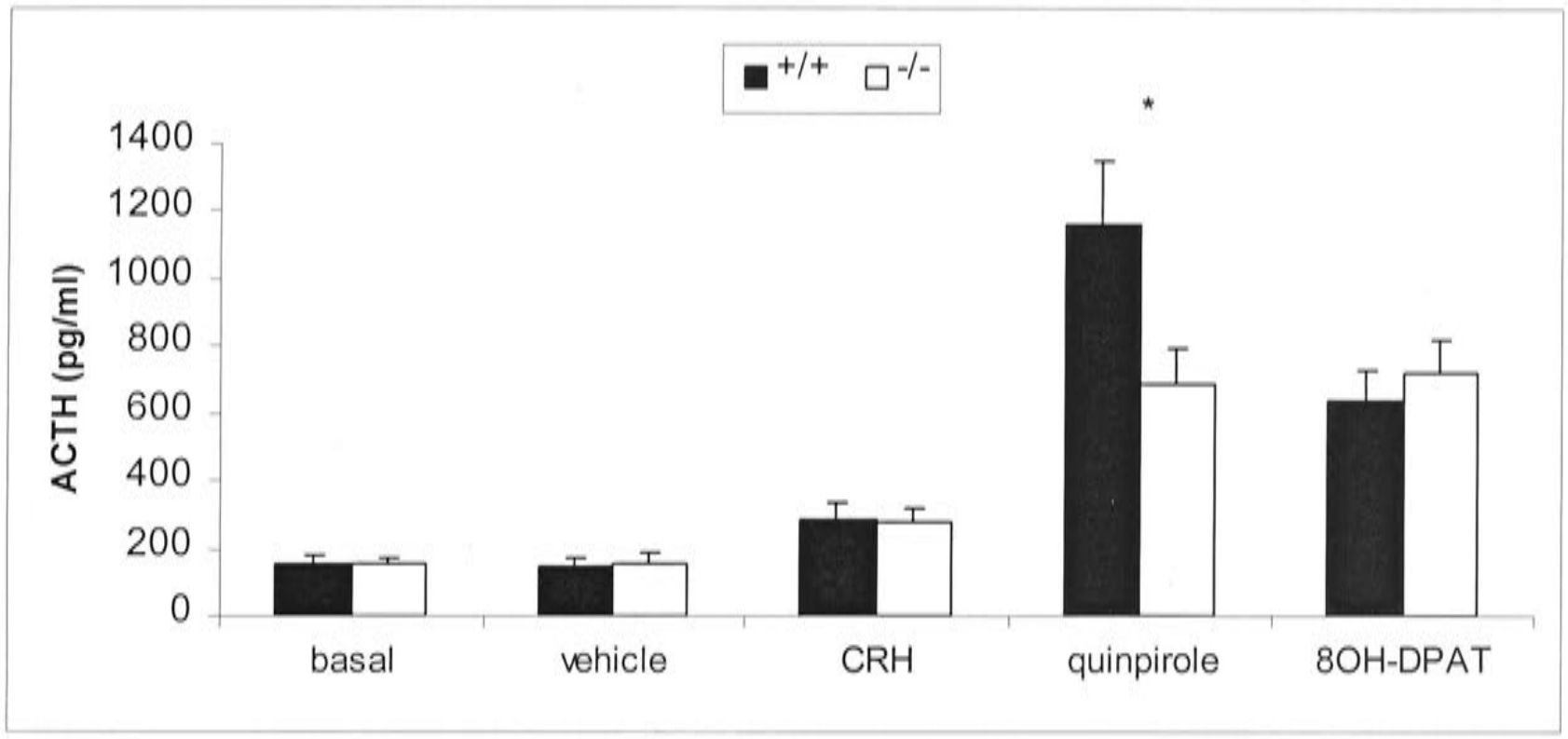


Figure 5.7: Quinpirole stimulation of plasma ACTH is significantly attenuated in the $G\alpha_z$ knockout mouse. Mice were injected with saline vehicle (8 +/+, 8 -/-), 10μg/kg corticotropin releasing hormone (CRH) (8 +/+, 8 -/-), 1mg/kg quinpirole (7 +/+, 6 -/-) or 0.5mg/kg 8-hydroxy-dipropylamino-tetralin (8OH-DPAT) (8 +/+, 9 -/-), and blood were collected via the retro-orbital route either 15 (all drugs except CRH) or 30 minutes (CRH) after the injection. ACTH levels in blood plasma were determined using a commercial RIA kit. The mean plasma ACTH levels ± SEM are shown. +/+ vs -/-: * $p<.05$.

specific antagonist, sulpiride (Borowsky and Kuhn, 1992). This quinpirole induced increase in ACTH level is also significantly attenuated in $G\alpha_z$ knockout mice (Genotype main effect: $F(1,25)= 5.36$, $p<.05$) (Figure 5.7). The mechanism by which dopamine agonists stimulate ACTH release remains obscure, but may depend on corticotrophin releasing hormone (CRH) secreted by hypothalamic neurons. As an additional control, we also examined CRH stimulated ACTH release. Intraperitoneal administration of 10μg/kg CRH produced a significant rise in plasma ACTH (Drug dose main effect: $F(1,28)= 12.5$, $p<.01$), as previously described (Muglia et al., 2000). However, there was no significant difference observed between wildtype and $G\alpha_z$ knockout mice (Genotype main effect: $F(1,28)<1$, NS) (Figure 5.7). The serotonin 1A receptor agonist, 8-hydroxy-dipropylamino-tetralin (8OH-DPAT), is known to stimulate plasma ACTH via hypothalamic CRH (Serres et al., 2000). As expected, 0.5mg/kg of 8OH-DPAT caused a significant rise in plasma ACTH fifteen minutes after the injection (Figure 5.7) (Drug dose main effect: $F(1,29)= 50.5$, $p<.001$). However, there was no difference between wildtype and $G\alpha_z$ knockout mice (Genotype main effect: $F(1,29)<1$, NS), suggesting the CRH receptor involved in ACTH secretion is not impaired in the $G\alpha_z$ knockout mouse.

5.2.7 Absence of compensation by other G protein α subunits

Finally, I examined whether the alteration of dopamine D2-like receptor function could be explained by changes in the expression levels of other G protein α or β subunits. Western blot analyses did not reveal compensatory changes in the levels of any of these proteins (Figure 5.8).

5.3 Discussion

Mice deficient in $G\alpha_z$ demonstrate hyperactivity to morphine (Figure 4.1 and 4.2) and to the psychostimulants, cocaine (Yang et al., 2000) and *d*-amphetamine (Figure 5.1). Since central dopaminergic pathways participate in the motor activating effects of morphine (Barrot et al., 1999; Cadoni and Di Chiara, 1999; Kalivas et al., 1983; Kalivas and Duffy, 1990) and these psychostimulants (Ushijima et al., 1995; van den et al., 1988; Xu et al., 1994) (Neisewander et al., 1995; Uhl et al., 2002; Xu et al., 2000b), I have examined the involvement of G_z in signaling mediated by dopamine receptors. G_z is a member of the inhibitory guanine nucleotide binding protein family. It is widely found in many regions of the brain where dopamine receptors are located, including the cerebral cortex, amygdala, caudate nucleus, putamen, nucleus accumbens, substantia

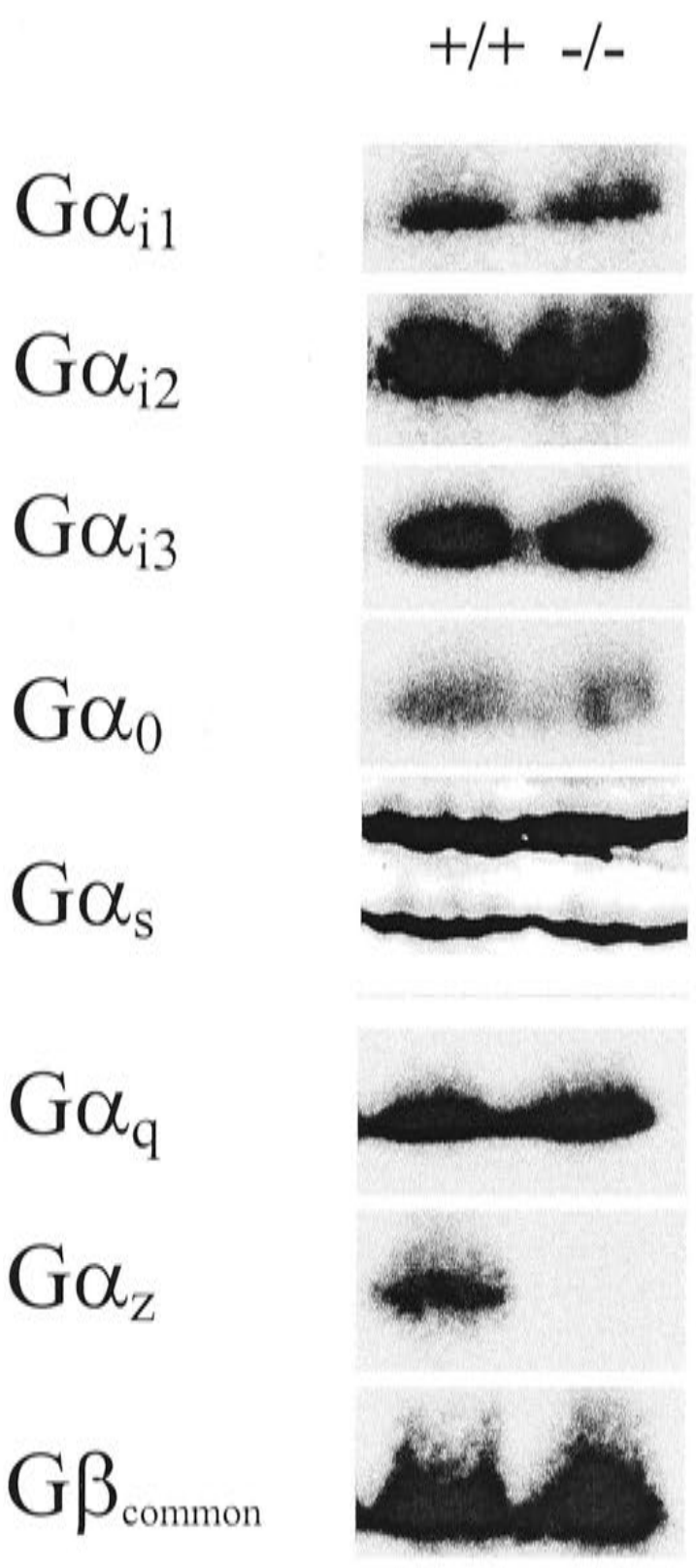


Figure 5.8: Levels of $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_{o}$, $G\alpha_{s}$, $G\alpha_{q}$, $G\alpha_{z}$, and $G\beta$ in the brains from wildtype and $G\alpha_{z}$ knockout mice. The proteins from the brains of wildtype and $G\alpha_{z}$ knockout mice were analysed by SDS-polyacrylamide gel electrophoresis, electro-transferred onto nitrocellulose, and blotted with antibodies specific for $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_{o}$, $G\alpha_{s}$, $G\alpha_{q}$, $G\alpha_{z}$, and $G\beta_{common}$. The experiment was repeated twice. There was some slight variation in the intensity of the protein bands due to protein transfer and blotting (see also Figures 3.8 and 3.9). However, no discernible difference could be observed between any of the $G\alpha$ and $G\beta$ subunits.

nigra, hypothalamus and hippocampus (Glick et al., 1998; Hinton et al., 1990; Serres et al., 2000; Wang et al., 1998). *In vitro* experiments suggest that G_z can couple to many of the same receptors as $G_{i/o}$, including dopamine D2-like receptors (Ho and Wong, 1998; Sidhu and Niznik, 2000). The data presented here demonstrate for the first time, that G_z is coupled to D2-like receptors in multiple dopaminergic pathways *in vivo*.

In COS-7 cells, G_z derived from transfection of the $G\alpha_z$ subunit, has been shown to be capable of coupling to both the long and short isoforms of the D2 and D3 receptors, and the D4 receptor, with the D4 receptor demonstrating the strongest coupling (Obadiah et al., 1999). Interestingly, D4 receptor knockout mice are also more responsive to the locomotor stimulating effects of ethanol, cocaine and methamphetamine (Rubinstein et al., 1997). D4 receptors are predominantly found in cerebral cortex (particularly the pre-frontal cortex), amygdala, hypothalamus and pituitary (Oak et al., 2000). They are also present at moderate to low levels in the caudate–putamen and nucleus accumbens, respectively (Rivera et al., 2002). In the nucleus accumbens, their presence in the core region has not been investigated. However, in the shell region, the majority of D4 receptors appear to serve as presynaptic heteroreceptors (Svingos et al., 2000), where they regulate the release of other neurotransmitters such as glutamate (Tarazi et al., 1998). It is therefore less likely that the attenuated locomotor suppressive effects of quinpirole in the $G\alpha_z$ knockout mouse is due to impairment of D4 receptor function since the inhibition of locomotor activity by quinpirole has been widely attributed to activation of dopamine autoreceptors (Lappalainen et al., 1990; Van Hartesveldt et al., 1994; Wang et al., 2000; Zhuang et al., 2001).

Among other members of the D2-like receptor family, both D2 and D3 receptors have been proposed to be capable of functioning as autoreceptors to inhibit dopamine release. However, studies of D2 and D3 receptor knockout mice have found the D2 receptor to be prominent in this role (Benoit-Marand et al., 2001; L'hirondel et al., 1998; Rouge-Pont et al., 2002; Schmitz et al., 2001), with the D3 receptor making only a minor contribution (Joseph et al., 2002). While most of these studies have been performed in the dorsal striatum, D2 receptors are also present in the nucleus accumbens (both core and shell) (Bancroft et al., 1998; Le Moine and Bloch, 1996; Levant, 1998), and are likely to perform the same role. D3 receptors are also found in the core and shell regions of the nucleus accumbens (Bancroft et al., 1998). However, these may be postsynaptic receptors coupled to short-loop negative feedback pathways that exert an

inhibitory influence on dopamine release (Koeltzow et al., 1998). Therefore, a partial impairment in the function of the D2 autoreceptor and to a lesser likelihood, the D3 receptor can contribute to a decrease in electrically evoked dopamine release produced by quinpirole in the $G\alpha_z$ knockout mouse. An alternative explanation of the data presented in Figure 5.5 is that the dopamine released by electrical stimulation was acting more efficiently on D2 autoreceptors in $G\alpha_z$ knockout animals, therefore partially occluding the effects of quinpirole added on top of natural dopamine. However, this explanation is unlikely as D2 autoreceptors in $G\alpha_z$ knockout animals are not fully saturated and can cause further depression of dopamine release with higher quinpirole doses (data not shown).

On the other hand, the hypothermic and hypolocomotor effects induced by D2-like agonists have been attributed solely to the D2 receptor, as these are abolished in the D2 receptor knockout mouse and retained in mice that lack the D3 receptor (Boulay et al., 1999a; Boulay et al., 1999b). The present observations of an attenuation in both the hypothermic and locomotor depressing effects of quinpirole suggest that G_z is coupled to D2 receptors to mediate these effects. Since these effects are not completely abrogated, the results also suggest that D2 receptors can couple to more than one G protein *in vivo*. However, whether the D2 receptors represent a single homogeneous receptor type is unclear. There is now evidence that the D2 receptor can form homomers among themselves and heteromers with other receptors in neurons (Franco et al., 2000; Lee et al., 2000). These D2 receptor homomers and heteromers would also be abolished in the D2 receptor knockout animal, and are therefore possible candidates for coupling to G_z to mediate the hypothermic and hypolocomotor effects of D2-like agonists. In addition, there are also two alternatively spliced forms of the D2 receptor. Mice that lack the long isoform of the D2 receptor have been successfully produced. The locomotor suppressive effects of quinpirole, as well as the autoreceptor function of the D2 receptor are not affected in these mice (Rouge-Pont et al., 2002; Usiello et al., 2000; Wang et al., 2000). This suggests that G_z is not coupled to the D2-long receptor.

The current results also suggest that the loss of G_z cannot be compensated by the presence of other G protein α subunits. Since G_o can couple to the D2 receptor (Jiang et al., 2001) and is probably found in all neurons, this inability of G_o to compensate for the absence of G_z is consistent with the existence of cellular compartments in intact cells, which restrict the pool of G proteins to which a receptor can gain access (Allgeier et al., 1997). It is not known whether G_z is localized in the same membrane compartment as G_o, as a consensus binding motif implicated in caveolin binding (Ostrom et al., 2000), is present in $G\alpha_o$ and most other $G\alpha_i$ family members, but is absent in $G\alpha_z$. Furthermore, the localization of G_z could also depend on the particular scaffold proteins expressed in the G_z containing cell, as scaffold proteins such as caveolin are not found in all cells (Galbiati et al., 1998). Moreover, the $\beta\gamma$ dimer that $G\alpha_z$ associates with can also

influence the targeting of $G\alpha_z$ and the affinity of G_z for receptors (Vanderbeld and Kelly, 2000). Whether $G\alpha_o$ and $G\alpha_z$ share the same $\beta\gamma$ subunits or $\beta\gamma$ subunits with similar biochemical properties is presently unknown.

Finally, G_z is also involved in mediating the secretion of ACTH caused by activation of D2-like receptors. This effect is believed to occur via dopamine receptors in the hypothalamus (Borowsky and Kuhn, 1992). Cocaine, an indirect dopamine agonist at both D1 and D2-like receptors, has also been found to stimulate ACTH release (Rivier and Vale, 1987). The mechanism involves CRH from the hypothalamus as the effect of cocaine can be blocked by a CRH antiserum (Rivier and Vale, 1987). Quinpirole induced ACTH release may also operate via a similar mechanism and there is recent evidence that stimulation of cortical CRH receptors results in the incorporation of a photo-reactive form of GTP, [α-^{32}P]-GTP-γ-azidoanlide into $G\alpha_z$ (along with $G\alpha_s$, $G\alpha_i$, $G\alpha_{q/11}$, $G\alpha_o$) (Grammatopoulos et al., 2001). As such, the present study examined whether G_z is coupled to CRH receptors to mediate ACTH release. My data did not reveal any significant difference between wildtype and $G\alpha_z$ knockout mice in CRH stimulated ACTH secretion. Additionally, stimulation of ACTH released by the serotonin 1A agonist, 8-hydroxy-dipropylamino-tetralin (8OH-DPAT), which is known to occur via a CRH mechanism (Serres et al., 2000), also did not differ between mice of the two genotypes. This suggests that CRH receptors in the pituitary, and serotonin 1A receptors in the hypothalamus that mediate ACTH secretion, are not coupled to G_z. This is in contrast to the findings of Serres and colleagues (2000) where the employment of $G\alpha_z$ antisense oligonucleotides was found to partially counteract the rise in plasma ACTH levels caused by 8OH-DPAT in rats. The difference may be attributed to species differences, general oligonucleotide toxicity or non-specificity in their $G\alpha_z$ antisense, which they have alluded to in one of their experiments. Alternatively, it is also possible that there are subtle developmental compensation in the $G\alpha_z$ knockout mouse that prevents the phenotype from being expressed. Nonetheless, taken together, these results suggest that pituitary CRH receptors are not impaired in the $G\alpha_z$ knockout mouse.

One common criticism of gene knockout studies is that there may be compensations in other parts of the system as a result of inactivation of the gene. However, my results clearly show that the absence of $G\alpha_z$ cannot be compensated for by $G\alpha_o$ and other more

abundant $G\alpha_i$ family members in the dopaminergic pathways examined. Such 'compensation' is more likely to be observed when receptor G protein coupling is studied in broken membrane preparations. The 'compensation' observed can be an artifact that results from disruptions to membrane compartments and scaffolds, which play an important role in determining signaling specificity *in vivo* (Hur and Kim, 2002; Tsunoda and Zuker, 1999). Indeed, in membranes prepared from rat RINm5F neuroendocrine cells, the galanin and somatostatin receptors showed promiscuous coupling to various G_i and G_o isoforms (Degtiar et al., 1997; Schmidt et al., 1991). In contrast, when the G protein coupling of the galanin and somatostatin receptors was examined in whole RINm5F cells by electrophysiology, the galanin receptor coupled specifically to G_{o1} and the somatostatin receptor to G_{o2} (Degtiar et al., 1997) to inhibit calcium channels. Similarly, in dog thyroid membranes, stimulation of the thyrotropin receptor was found to cause the incorporation of [α-^{32}P]-GTP-γ-azidoanlide into $G\alpha_s$, $G\alpha_i$, and $G\alpha_{q/11}$, suggesting that activation of the thyrotropin receptor would normally stimulate these G proteins (Allgeier et al., 1997). However, in the same experiment, when intact thyrocytes were used, the thyrotropin receptor could only couple to G_s and G_i (Allgeier et al., 1997). This indicates that although the thyrotropin receptor can recognize $G_{q/11}$ in disrupted membranes, membrane compartmentalization in an intact cell prevents the receptor from interacting with $G_{q/11}$ *in vivo*. Consistent with this idea, there is now evidence that G_s, G_i, and $G_{q/11}$ may be targeted to distinct microdomains within the plasma membrane of a cell (Oh and Schnitzer, 2001), which is likely to affect their interactions with different receptors.

In summary, I have demonstrated that G_z can couple to D2-like receptors *in vivo*. Since disturbances in dopaminergic networks are implicated in a number of debilitating human conditions and diseases, including Parkinson's disease, schizophrenia, and drug addiction, G_z might serve as a potential drug target when subtle fine tuning of dopaminergic functions are required.

Chapter 6

The regulation of body temperature by opioids in the $G\alpha_z$ deficient mouse

6.1 Introduction

Morphine is a powerful analgesic. It is widely employed to successfully relieve pain during and after surgery, but also produces a number of undesirable side effects. One perioperative side effect is the alteration of body temperature (Su et al., 1987). Perioperative hypothermia can result in complications such as myocardial ischemia (Frank et al., 1993), reduced resistance to wound infection (Kurz et al., 1996) and impaired coagulation of blood platelets (Schmied et al., 1996). Understanding and preventing this and other side effects of morphine is therefore one of the challenges of modern medical science.

Morphine produces all its effects through opioid receptors in our body. There are three main classes of opioid receptors, and they have been designated using the Greek letters, μ, δ and κ (Kieffer, 1999). Morphine binds to the μ receptor with the highest affinity, and to the δ and κ receptor with lower affinities (Goldstein and Naidu, 1989; Raynor et al., 1994). The effects of morphine on body temperature are thought to be mediated primarily through the μ and κ receptors, with the δ receptor playing an insignificant role (Chen et al., 1996; Sarton et al., 2001).

Following morphine binding, opioid receptors become activated and the activation signals are transduced through heterotrimeric G proteins. The G protein(s) responsible for transducing the thermoregulatory effects of morphine are currently unknown. However, μ receptors in the brain have been found to be capable of coupling to the G proteins G_z, G_{i2} and G_o *in vivo* (Garzon et al., 1997b; Garzon et al., 1997a; Garzon et al., 1998; Jiang et al., 2001). On the other hand, the κ receptor can couple to G_s in cultured neurons (Hampson et al., 2000) and to pertussis toxin sensitive $G_{i/o}$ proteins in the brain (Dar, 1998; Konkoy and Childers, 1993). However, there is only *in vitro* evidence that the κ receptor can couple to G_z when both proteins were transfected into human embryonic kidney 293 cells (Lai et al., 1995).

In Chapter 3, I have demonstrated that supraspinal morphine analgesia is impaired in the $G\alpha_z$ knockout mouse after chronic morphine treatment, suggesting that G_z is involved in transducing the analgesic effects of morphine. In this Chapter, my aim is to examine whether the thermoregulatory effects of morphine are also affected by the loss of G_z.

6.2 Results

6.2.1 Stress induced hyperthermia

It is well known that the repeated use of a rectal probe to measure body temperature causes stress induced hyperthermia in rodents (van der Heyden et al., 1997). Since the experimental procedure requires multiple temperature measurements to be made in the same mouse, I began by analysing how the body temperature of mice changes with repeated measurements. The results reveal a tendency for the $G\alpha_z$ knockout mouse to exhibit a slightly higher body temperature. However, the difference in body temperature between mice of the two genotypes was not statistically significant (Genotype main effect: $F(1,57)= 2.55$, NS). Repeated use of the rectal probe caused a sharp rise in body temperature that is similar in both wildtype and $G\alpha_z$ knockout mice (Genotype main effect: $F(1,57)= 1.17$, NS), which peaked and stabilized after the second measurement (Figure 6.1). Therefore, to calculate the pre-drug body temperature baseline for each mouse, the first reading was discarded and the last three readings recorded immediately before drug (or vehicle) administration were averaged.

6.2.2 Morphine induced hypothermia is attenuated in the $G\alpha_z$ knockout mouse.

When wildtype and $G\alpha_z$ knockout mice were administered morphine, there was a slight hyperthermia at the low morphine dose of 3mg/kg (Drug dose main effect: $F(1,34) = 12.4$, $p<.002$) and considerable hypothermia at higher doses (Chen et al., 1996; Geller et al., 1983). The hypothermic effect of morphine was found to depend on genotype (Genotype by Dose interaction: $F(2,36)= 25.2$, $p<.001$), such that mice deficient in $G\alpha_z$ showed a significantly attenuated response (Figure 6.2).

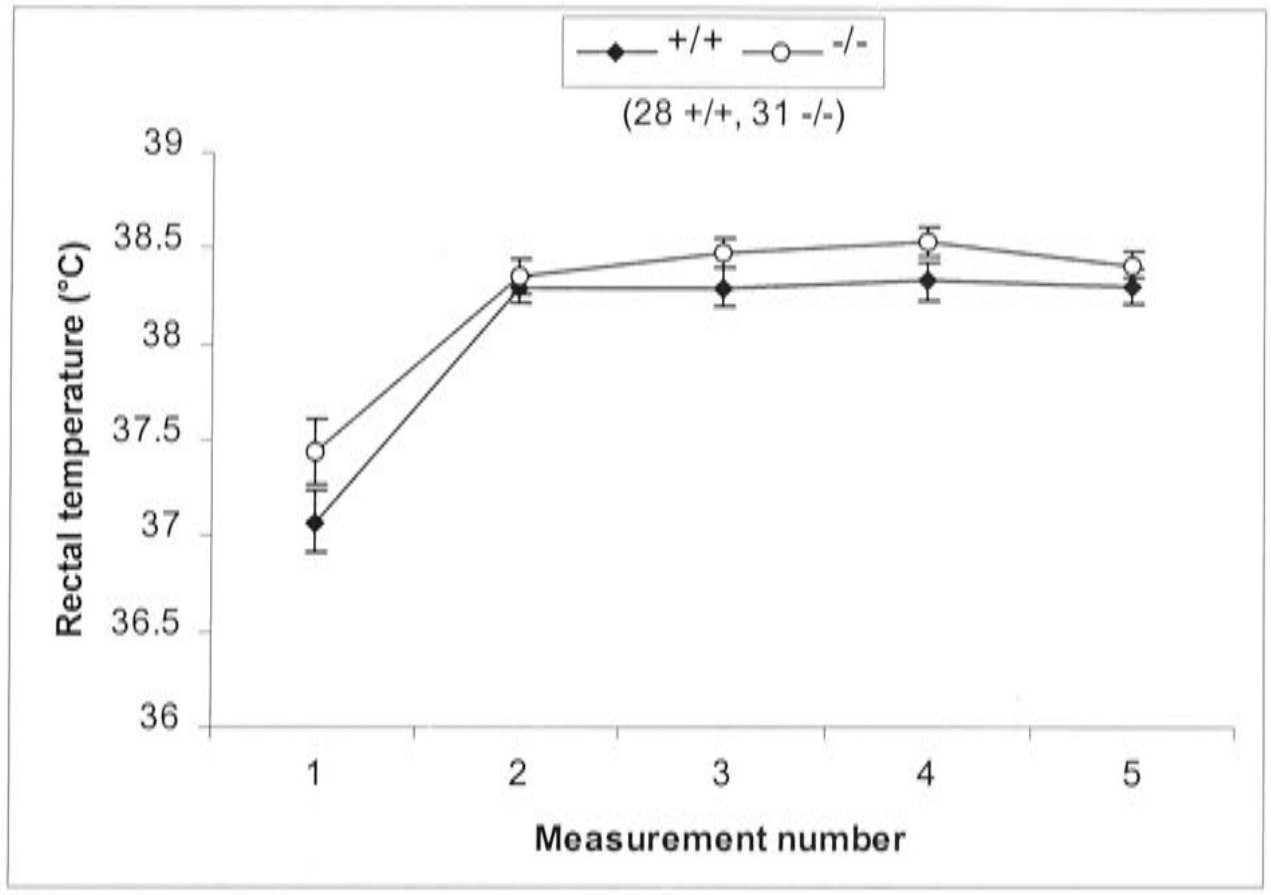


Figure 6.1: Body temperature in mice after repeated measurements. The body temperature of wildtype and $G\alpha_z$ knockout mice were measured at 15 minute intervals using a rectal probe.

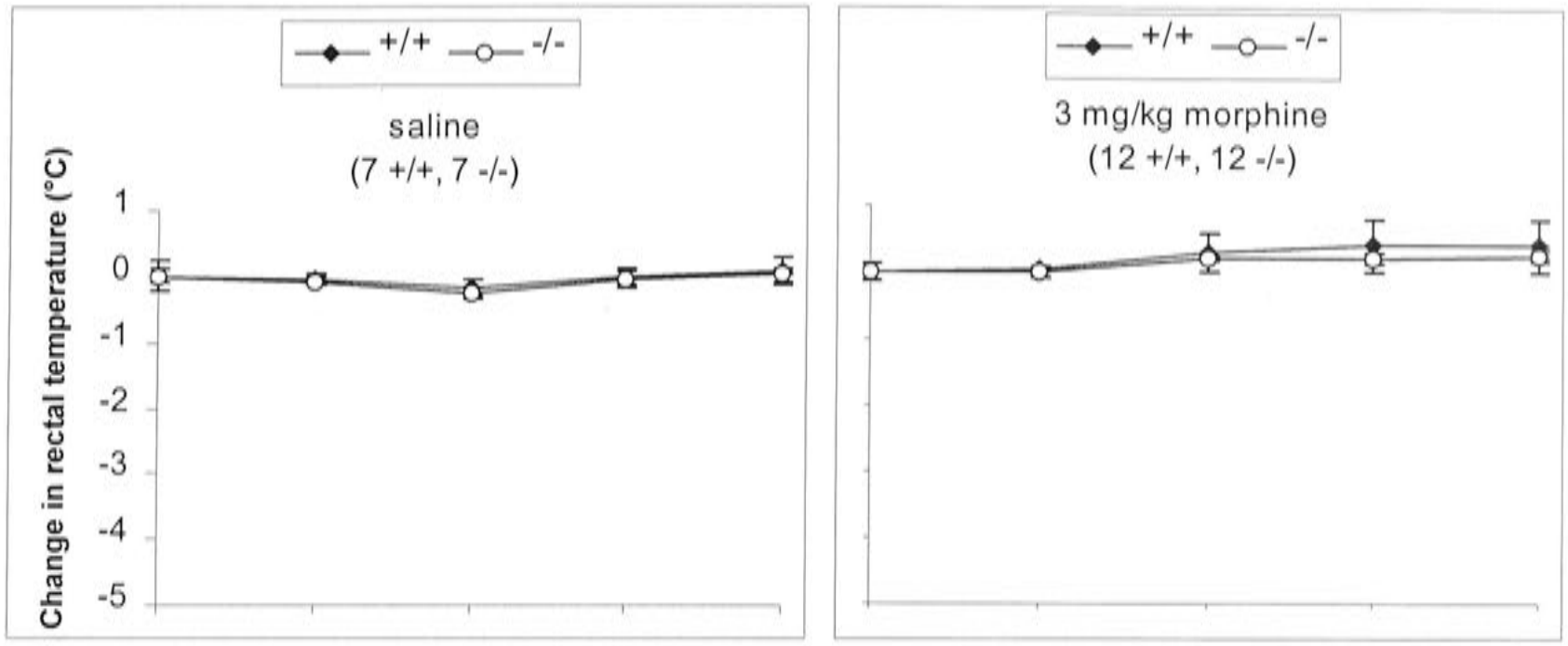


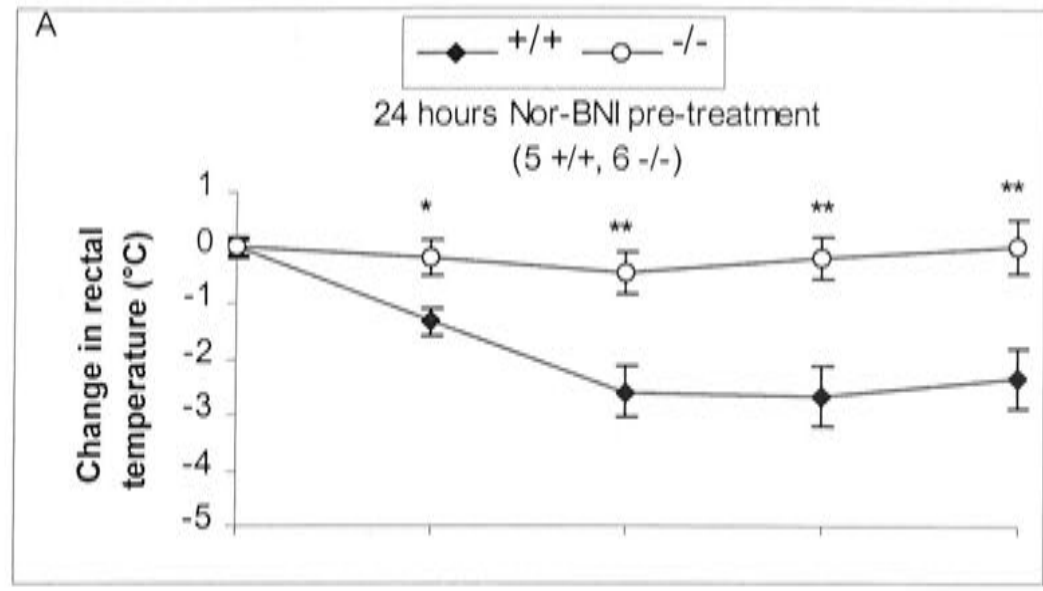
Figure 6.2: Attenuation of the thermic effects of morphine in the $G\alpha_z$ knockout mouse. Wildtype and $G\alpha_z$ knockout mice were injected subcutaneously with either saline vehicle, 3mg/kg, 30mg/kg or 100mg/kg morphine hydrochloride at time 0, and body temperature was measured every 15 minutes after morphine administration for 1 hour. The plotted values are means ± SEM. *p<.05, **p<.01, ***p<.001

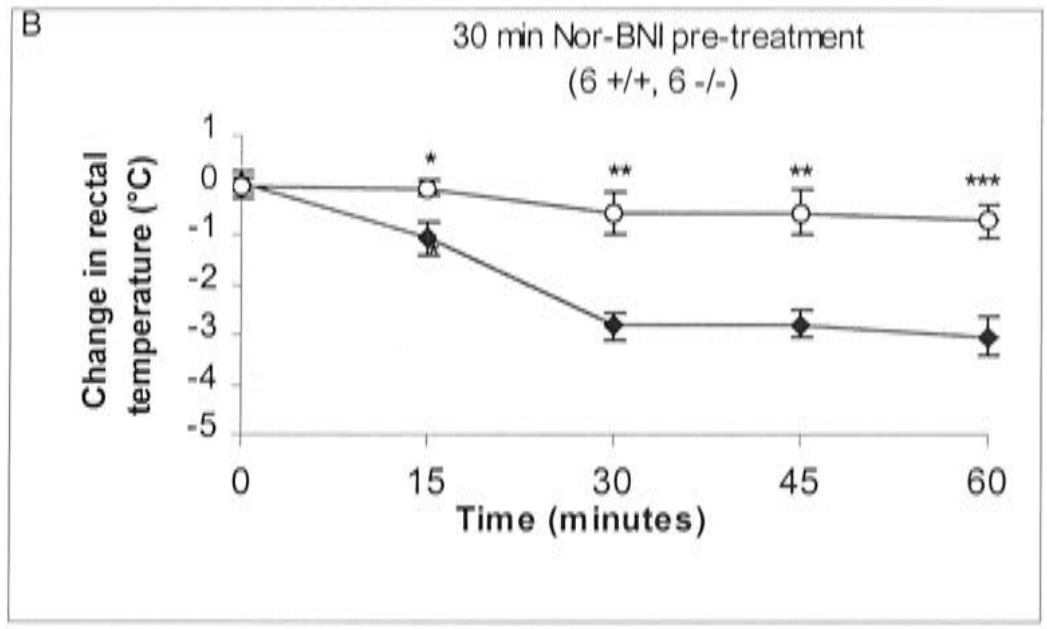
6.2.3 The κ opioid receptor antagonist, Nor-BNI, does not affect morphine induced hypothermia

Since there is evidence that the hypothermic effect of morphine at high doses may be attributed to morphine stimulation of the κ opioid receptor (Chen et al., 1996), the role of the κ receptor in morphine induced hypothermia was next examined. Mice were pre-treated for 24 hours with 20mg/kg of the κ receptor antagonist, nor-binaltorphimine (Nor-BNI). This dose and pre-treatment time period were chosen based on previous work showing its effectiveness in preventing the analgesic and thermic effects of κ receptor activation in mice (Endoh et al., 1992). The nor-binaltorphimine treated mice were then tested with 30mg/kg morphine 24 hours later. The data obtained shows that nor-binaltorphimine pre-treatment does not significantly affect morphine induced hypothermia in wildtype mice (Drug main effect: $F(1,11)=0.011$, NS) (Figure 6.3). Similar data was obtained when the nor-binaltorphimine pre-treatment time period was shortened to 0.5 hour (Drug main effect: $F(1,12)= .234$, NS) (Figure 6.3).

6.2.4 Activation of the κ opioid receptor does not produce hypothermia in C57/BL6 mice

Since pre-treatment with nor-binaltorphimine has no effect on the hypothermic response produced by morphine, it may be possible that activation of the κ receptor does not cause hypothermia in the C57BL/6 mouse strain. Indeed, there is evidence of variations in responses between mouse strains to stimulation of the κ receptor (Itoh et al., 1993; Thorat et al., 1993). To test this hypothesis, the thermic response of wildtype and $G\alpha_z$ knockout mice to 30mg/kg of the κ receptor agonist, U50,488H was measured. This high dose of U50,488H has previously been found to induce significant hypothermia in Swiss-Webster mice (Thorat et al., 1993), but not in mice of the ddY strain (Itoh et al., 1993). When wildtype and $G\alpha_z$ knockout mice were treated with 30mg/kg U50,488H, severe motor retardation was observed (see Figure 4.6 for the locomotor inhibitory effects of 3mg/kg and 10mg/kg U50,488H), indicating that the drug was producing an effect. However, body temperature was not significantly affected in either wildtype or $G\alpha_z$ knockout mice (Drug main effect: $F(1,22)=0.051$, NS) (Figure 6.4). Therefore, similar to the ddY mouse, activation of the κ receptor does not induce significant hypothermia in C57BL/6 mice. The decrease in hypothermic response in the $G\alpha_z$





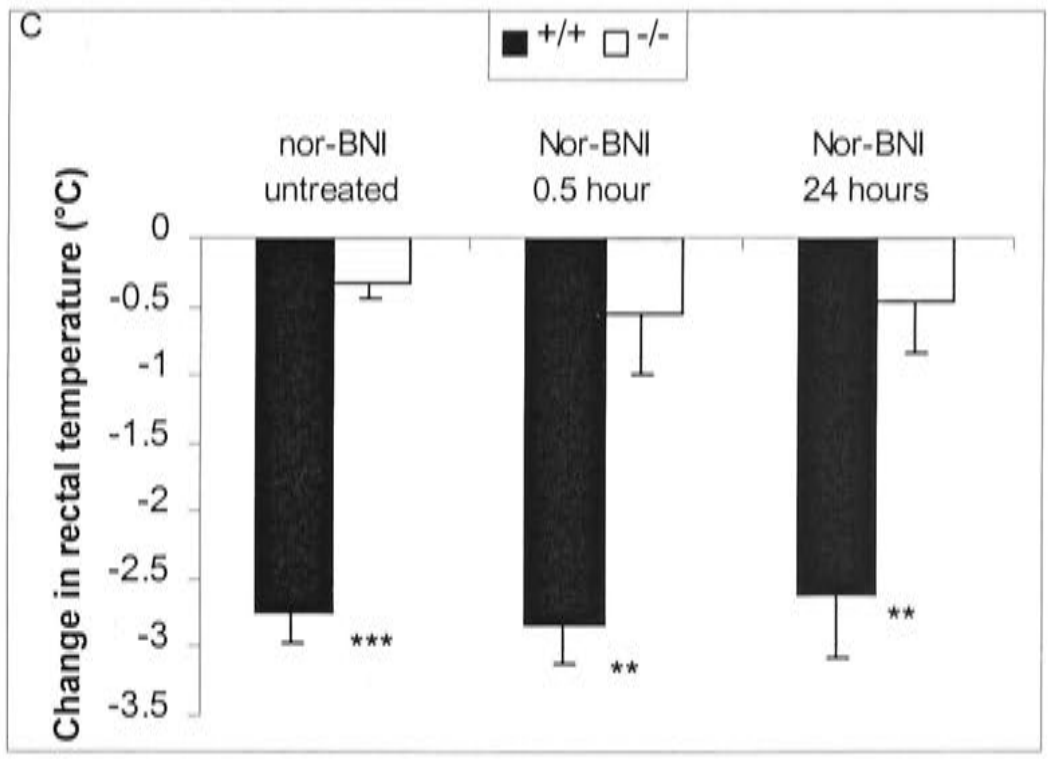


Figure 6.3: Blocking of the κ opioid receptor does not significantly affect the hypothermic response produced by morphine. Wildtype and $G\alpha_z$ knockout mice were administered 20mg/kg of the κ receptor antagonist, nor-binaltorphimine (Nor-BNI) either (A) 24 hours or (B) 0.5 hours prior to morphine treatment. Following the injection of 30mg/kg morphine hydrochloride, the body temperature of the mice was measured every 15 minutes for 1 hour (C) The change in rectal temperature of untreated and nor-BNI pre-treated mice, 30 minutes after 30mg/kg morphine hydrochloride. *p<.05, **p<.01, ***p<.001

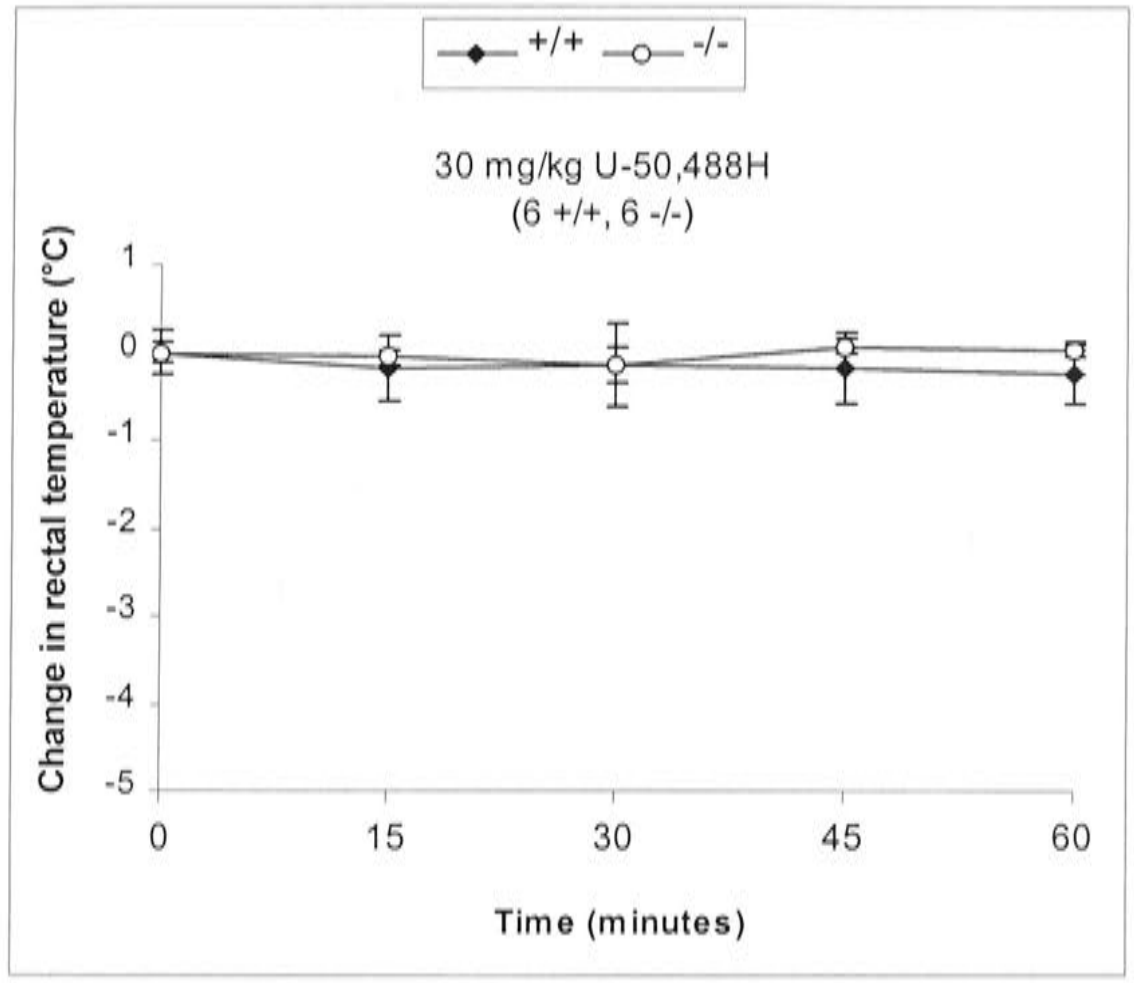


Figure 6.4: Activation of the κ opioid receptor by U50,488H does not alter the body temperature of wildtype and $G\alpha_z$ knockout mice. Wildtype and $G\alpha_z$ knockout mice were injected subcutaneously with 30mg/kg of the κ receptor agonist, U50,488H. The body temperature of the mice was then measured every 15 minutes for 1 hour.

mouse is therefore, unlikely to be due to an impairment of κ receptor function.

6.3 Discussion

The results presented in this chapter clearly demonstrate the involvement of $G\alpha_z$ in mediating the thermoregulatory effects of morphine. At a dose of 30mg/kg, morphine does not significantly alter the body temperature of $G\alpha_z$ knockout mice, while producing hypothermia in wildtype mice. When the morphine dose is increased to 100mg/kg, both wildtype and $G\alpha_z$ knockout mice show hypothermia, but the response is very much attenuated in the mutant mice.

The receptor pathway(s) affected by the absence of $G\alpha_z$ to cause the observed reduction in the hypothermic response to morphine is currently obscure. In rats, the biphasic thermoregulatory effect of morphine is known to be mediated by two different opioid receptors. Low doses of morphine produced hyperthermia through the μ receptor while high doses caused hypothermia through the κ receptor (Chen et al., 1996). This is consistent with findings in the literature, where stimulation of the μ receptor mediates hyperthermic responses while activation of the κ receptor produces hypothermia in rats (Adler and Geller, 1993).

In mice, the picture appears more complicated. A survey of fifteen mouse strains found large strain variations in their thermic responses to morphine, with several strains responding only with hypothermia, some strains showing the same biphasic response as rats and one strain failing to respond across all morphine doses tested (from 4mg/kg to 32mg/kg) (Belknap et al., 1998). While stimulation of the μ receptor appears to elicit either a biphasic (Rosow et al., 1980) or hypothermic response in mice (Baker and Meert, 2002), activation of the κ receptor results in different responses across mouse strains. For instance, the κ receptor agonist U50,488H produced a biphasic response in NMRI mice (from 2.5mg/kg to 40mg/kg) (Baker and Meert, 2002), but only hypothermia in Swiss-Webster mice (from 8mg/kg to 32 mg/kg) (Bhargava and Thorat, 1993). In ddY mice, U50,488H produced hyperthermia at 5mg/kg (Endoh et al., 1992) and no significant thermic effect at 30mg/kg (Itoh et al., 1993). Although some of these disparities may be attributed to differences in experimental conditions between

laboratories such as ambient temperature, mouse handling and perhaps the dose of the drug, it is clear that at least part of the response variation is contributed by idiosyncrasies between mouse strains. It is also evident that mice do not respond to U50,488H in the same way as rats since U50,488H can cause hyperthermia in mice (Endoh et al., 1992), but it only produces hypothermia in rats (Adler and Geller, 1993). It is important therefore to be careful when extrapolating results from rats to mice and across different mouse strains.

In 129/SV:C57BL/6 F2 mice, morphine induced a hypothermic response across a very wide dose range (6mg/kg to 100mg/kg), which was completely abolished in mice lacking the μ opioid receptor (Sarton et al., 2001). This suggests an essential role of the μ receptor in mediating the hypothermic response to morphine. The present observation of considerable hypothermia caused by 30mg/kg and 100mg/kg morphine in wildtype C57BL/6 mice may therefore be due to morphine activation of the μ receptor. If this is the case, the significant attenuation of the hypothermic response in the $G\alpha_z$ knockout mouse may indicate an impairment of μ receptor function. This in turn, would suggest that the μ receptor is normally coupled to G_z to mediate hypothermia in this mouse strain. Since many of the agonists selective for the μ receptor are peptides, a test of this hypothesis will require direct intracerebroventricular injection of the peptide agonist into the mouse brain (which my laboratory is currently still trying to set up to do). In addition, one could also employ a μ selective antagonist to check whether it will prevent the morphine induced hypothermic response in wildtype C57BL/6 mice.

Alternatively, it is possible that the attenuation of morphine hypothermia in the $G\alpha_z$ knockout mouse is due to some alteration of neurotransmission downstream from the opioid receptors. There is evidence that dopamine and adenosine receptors may be involved in the hypothermia induced by morphine in Swiss albino mice. Pre-treatment with the adenosine receptor antagonist, theophylline and the dopamine D2-like receptor antagonists, sulpiride and pimozide (but not the dopamine D1-like receptor antagonist SCH 23390) caused a dose dependent decrease in the hypothermic response to morphine (Zarrindast et al., 1994). Both the adenosine A1 and dopamine D2-like receptors are capable of coupling to G_z *in vitro* (Obadiah et al., 1999; Wong et al., 1992). Furthermore, I have demonstrated that the hypothermic response induced by the D2-like receptor agonist, quinpirole, is significantly attenuated in the $G\alpha_z$ knockout

mouse (Chapter 4), suggesting that D2-like receptors in the thermoregulatory pathway are probably coupled to G_z. Accordingly, an impairment of D2-like receptor function in the $G\alpha_z$ knockout mouse may also contribute to the observed attenuation of morphine hypothermia.

In conclusion, this chapter demonstrates that morphine induced hypothermia, a major undesirable side effect of morphine during its surgical use, is significantly attenuated in mice lacking $G\alpha_z$, and therefore suggests a potential site for therapeutic clinical intervention. However, the mechanism underlying this phenomenon is currently restricted to this mouse strain and whether the inactivation of $G\alpha_z$ mediated signal transduction pathways can prevent morphine hypothermia in other mouse strains and humans would need to be further explored.

Chapter 7

General Discussion

In this thesis, I have examined the consequences of disrupting the gene that codes for $G\alpha_z$, which results in the absence of signalling through the heterotrimeric G protein, G_z, in mice. I have presented evidence suggesting that G_z signalling is important in the development of tolerance to the analgesic and lethality effects of morphine, such that in $G\alpha_z$'s deficiency, greater tolerance develops. I have also employed a number of converging lines of evidence suggesting for the first time that G_z is coupled to D2-like receptors *in vivo*. This impairment of D2-like receptor function in the nucleus accumbens, may explain the enhanced stimulation of locomotor activity by psychostimulants and morphine in the mutant mouse. I have also demonstrated that G_z signalling is involved in the hypothermic response to morphine. Physical dependence on morphine is also altered in the mutant mouse, suggesting the participation of G_z mediated pathways in the opioid withdrawal phenomenon.

7.1 Compensation at the molecular level

7.1.1 Compensation by upregulation of another G protein

One common criticism of gene knockout studies is the issue of compensation by other proteins for the inactivation of the protein encoded by the disrupted gene (Nelson, 1997). At the level of gene transcription, this compensation can happen when a number of genes are co-regulated together. I have examined the expression of $G\alpha_s$, $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_o$ and $G\alpha_q$ in the brains of $G\alpha_z$ mutant mice, and my results did not reveal any noticeable change in the quantities of these other G protein α subunits (Figure 5.8). This result is in agreement with a similar lack of compensation of other G protein α subunits in the $G\alpha_o$ knockout mouse (Mende et al., 1998), but differs from the observation of a compensatory increase in $G\alpha_{i3}$ in the $G\alpha_{i2}$ knockout mouse (Rudolph et al., 1996), indicating that the expression levels of some G protein α subunits are regulated independently while others are not.

7.1.2 Compensation by functional redundancy between G proteins

In the absence of upregulation, other G protein α subunits may still compensate for the absence of $G\alpha_z$ at the functional level. $G\alpha_z$ is expressed at a relatively low level compared to $G\alpha_i$ and $G\alpha_o$ in the brain [2] (Sternweis and Robishaw, 1984) and *in vitro* experiments have shown $G\alpha_{i/o}$ to be capable of coupling to the same receptors and effectors as $G\alpha_z$ (Ho and Wong, 1998). Accordingly, one may expect the deficiency of $G\alpha_z$ at the receptor, to be easily compensated by the presence of $G\alpha_{i/o}$, since $G\alpha_{i/o}$ are present in substantial excess compared to the number of receptors and effectors (Milligan, 1996). Functionally, this compensation may possibly have occurred to a certain degree, as evidenced by the small difference between the acute analgesic effects of morphine in wildtype and $G\alpha_z$ knockout mice (Figure 3.2a), despite evidence suggesting that G_z is the 'preferred' G protein at morphine receptors (Garzon et al., 1998). Nonetheless, the consequences of the absence of $G\alpha_z$ can still be felt as suggested by the shortened duration of the analgesic response in the $G\alpha_z$ knockout mouse (Figure 4.7).

Functional compensation by other G protein α subunits may also have occurred during development. Although $G\alpha_z$ knockout mice are smaller than the wildtype control mice at weaning, the difference seems to disappear in adult mice (Figure 7.1). Moreover, no gross morphological abnormalities have been observed in either the brain or in other parts of the $G\alpha_z$ knockout mouse, despite evidence suggesting that $G\alpha_z$ may be involved in developmental signalling (Chapter 1, Sections 1.5.2, 1.5.3, 1.5.4). Since the molecular targets of $G\alpha_z$ during development are shared with other $G\alpha_{i/o}$ family

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[2] Due to the differential affinity of antibodies for proteins on Western blots, a direct comparison of the amount of different G protein α subunits present in the brain cannot be made without the use of known levels of pure recombinant α subunits as standards. However, Sternweis and Robishaw (1984) have provided data showing that $G\alpha_i$ and $G\alpha_o$, together, accounted for half of the total GTPγS binding to all guanine nucleotide binding proteins in brain membranes. Since guanine nucleotide binding proteins include not only heterotrimeric G proteins, but also a large number of other GTPases (see Chapter 1, section 1.1.2), the data suggests that $G\alpha_i$ and $G\alpha_o$ exist in large quantities relative to other G protein α subunits in the brain. Indeed, Asano et al.. (1988) found $G\alpha_o$ alone to account for 0.5% of all brain membrane proteins. Another piece of evidence comes from the study of the expression of G_β subunits. The level of expression of the G_β protein is known to be co-regulated with the α subunit as the absence of $G\alpha_o$ caused the amount of G_β subunits in the brain to be reduced to just 32% of the level in wildtype brains (Mende et al., 1998). Similarly, in $G\alpha_{i2}$ deficient embryonic fibroblasts, there is a parallel reduction of G_β to 50% of wildtype levels (Rudolph et al., 1996). In contrast, the loss of $G\alpha_z$ did not result in any detectable change in the quantity of G_β subunits (Figures 3.8 and 5.8). This suggests that the overall level of expression of $G\alpha_z$ is low compared to the sum total of all G protein α subunits in the brain.

members (Chapter 1, Sections 1.5.2, 1.5.3, 1.5.4), it is likely that some form of compensation by other $G\alpha_{i/o}$ proteins has occurred. For instance, nerve growth factor (NGF) dependent neuronal survival is decreased in $G\alpha_z$ deficient sympathetic neurons only after the addition of pertussis toxin, suggesting that $G\alpha_{i/o}$ can compensate for the absence of $G\alpha_z$ in this system (Powell et al., 2002). This kind of compensation may occur either through $G\alpha_{i/o}$ binding directly to the same molecular partners and effectors as $G\alpha_z$ or through its mediation of a signalling cascade that produce the same functional outcomes as activation of the G_z cascade.

7.1.3 Failure of compensation and its implications

Given the relative abundance of $G\alpha_{i/o}$ in the brain, it is surprising that functional compensation either does not occur or it occurs to an insignificant extent under most conditions examined in the present thesis. The observations in the $G\alpha_z$ knockout mouse of a marked attenuation in morphine induced hypothermia, as well as a significant reduction in the effects of D2-like receptor activation, suggest that in the signalling pathways mediating these other functions, the presence of $G\alpha_z$ is critical for normal signalling. As $G\alpha_{i/o}$ are very likely to be also expressed in the cells containing the 'affected' signal transduction pathways (given their ubiquitous and high expression level in the brain), the lack of significant compensation by $G\alpha_{i/o}$ provides illuminating insights about the organization of cellular signalling, as well as a possible mechanism of functional compensation.

7.1.3.1 Cellular signalling is compartmentalized in native cells

One question that needs to be answered concerns why $G\alpha_{i/o}$ can couple to most (if not all) $G\alpha_z$ receptors in transfected cells *in vitro* (Ho and Wong, 1998) but frequently fail to compensate for the absence of $G\alpha_z$ in native cells *in vivo*. An important clue to the answer of this question comes from a study comparing G protein activation in COS-7 cells that have been transfected with μ and δ opioid receptors, with SK-N-SH neuroblastoma cells, which naturally co-express the two receptors. In the artificially transfected cells, stimulation of either the μ or δ receptor activates a common pool of G proteins that is shared between the two receptors; while in native cells, each receptor

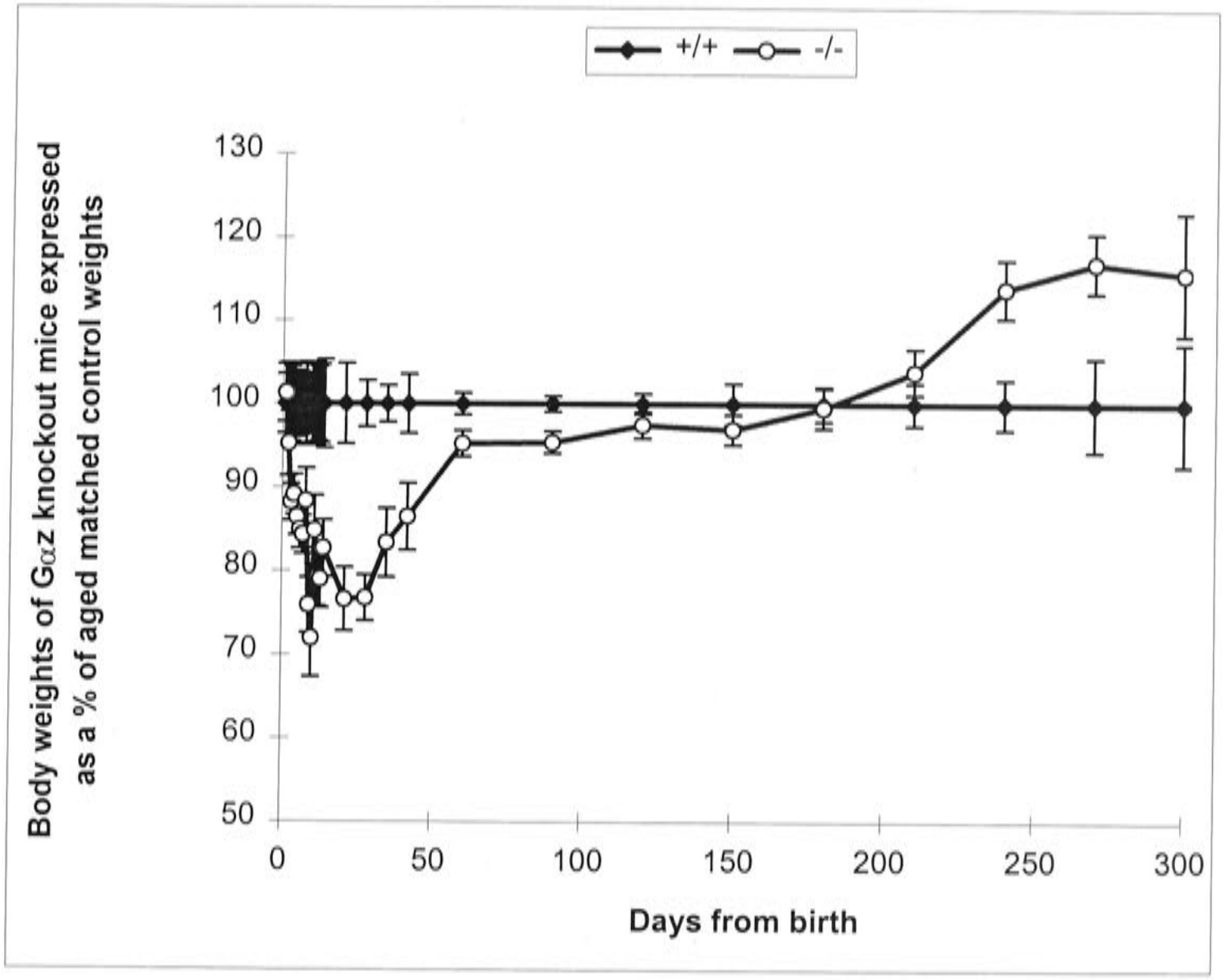

Figure 7.1: Developmental compensation in the $G\alpha_z$ knockout mouse. Wildtype and $G\alpha_z$ knockout mice from mainly heterozygous matings were weighed on various days after birth. The data for mice less than 2 months old were collected by Dr. Kim Kelleher while she was doing her Ph.D. The data for some of the adult mice were collected by myself in collaboration with her.

activates its own pool of G proteins. This occurs even though the two pools of G proteins in native cells contain $G_{i/o}$, which presumably share a similar capacity to couple to identical receptors (Shapira et al., 2000). To reconcile these observations, the authors proposed the existence of cellular compartments in native cells, which restrict the access of receptors to only those G proteins that lie in close proximity (Shapira et al., 2000). Consistent with this idea, there is now evidence that receptors, G proteins and effectors may be organized into large signalling complexes (Chidiac, 1998; Slesinger et al., 1995) with the help of scaffolding proteins (Ostrom et al., 2000; Scott and Zuker, 1998). This would help to explain the capacity of type V adenylate cyclase, a G protein effector, to enhance receptor mediated activation of G_i and G_s (Scholich et al., 1999; Wittpoth et al., 2000), as it can occur only if the three proteins (receptor, G protein and effector) lie in close association with one another. It would also explain the exclusive specificity of receptor-G protein-effector coupling observed in intact cells (such as those obtained by employing electrophysiological methods (Kleuss et al., 1993)), which were lost following membrane disruption (Allgeier et al., 1997; Degtiar et al., 1997). The findings also provide a much needed theoretical framework for understanding the rapidity and specificity of signalling processes in cells (Albert and Robillard, 2002).

7.1.3.2 Over-expression of receptors or G proteins can potentially disrupt cellular compartments leading to promiscuous signalling

Currently, the stoichiometry and composition of such signalling complexes are largely unknown. It may vary between different receptor and cell types, partially dependent on the level of expression of each component in the complex. Estimates of the number of G proteins to receptors in such complexes have ranged from one G protein to one rhodopsin receptor in drosophila photoreceptor cells (Scott and Zuker, 1998) to four G proteins to one μ opioid receptor in C6 glioma cells stably expressing the μ receptor (Remmers et al., 2000). Compared to native cells, the over-expression of receptors or G proteins may produce two potential problems, hinging on the level of over-expression. Firstly, since endogenous signalling complexes assemble with the help of scaffolding proteins, if the transfected receptor or G protein can bind to the scaffolding protein, there will be competition for these scaffolding proteins. As a result of this competition and over-crowding at the plasma membrane, the scaffolding proteins may no longer be effective in their role of segregating the cell into distinct signalling compartments. Consequently, receptors and G proteins that normally do not interact, may now come

into contact with one another. Next, the over-expression of receptors or G proteins may decrease the specificity of the over-expressed molecule in its interaction with other proteins. For instance, a receptor and a G protein that have a weak affinity for one another, may interact when over-expressed due to an abnormally high concentration of the over-expressed protein (Dumont et al., 2002). Accordingly, although *in vitro* studies have suggested that $G\alpha_z$ and $G\alpha_{i/o}$ share similar structural elements required for recognition by receptors and effectors, the caveats highlighted above emphasized the importance of verifying these interactions *in vivo*.

7.1.3.3 Other factors that contribute to the occurrence of functional compensation

Besides the capacity to bind to the same receptors and effectors, the ability of $G\alpha_{i/o}$ to compensate for the absence of $G\alpha_z$ *in vivo* depends on a number of other factors. These include whether $G\alpha_{i/o}$ and $G\alpha_z$ are targeted to the same region of the cell (e.g. axon terminal versus cell body) where '$G\alpha_z$ receptors and effectors' are located, as well as their capacity to share similar G protein $\beta\gamma$ subunits and scaffolding proteins, which are involved in receptor recognition (Gautam et al., 1998), and assembly of signalling complexes respectively (Albert and Robillard, 2002; Huang et al., 1997a; Milligan and White, 2001). To date, very little is known about the co-localization of $G\alpha_{i/o}$ and $G\alpha_z$, their $\beta\gamma$ partners, as well as the scaffolding proteins to which they may bind. However, a putative caveolin binding motif conserved among all $G\alpha_{i/o}$ is found to be slightly altered in $G\alpha_z$ (Ostrom et al., 2000). This may result in $G\alpha_z$ being targeted to a different cellular environment in some cells. Any of the above factors may explain the lack of compensation by $G\alpha_{i/o}$ in the $G\alpha_z$ knockout mouse.

7.1.3.4 A possible mechanism of functional compensation

If $G\alpha_{i/o}$ cannot replace $G\alpha_z$ in the assembly of the '$G\alpha_z$ signalling complex', how does functional compensation for the absence of $G\alpha_z$ take place ? One mechanism may be the existence of a reserve pool of '$G\alpha_{i/o}$ signalling complexes' that can bind to the same agonists and produce similar functional consequences as '$G\alpha_z$ signalling complexes' when the former are stimulated. These spare '$G\alpha_{i/o}$ signalling complexes' may then compensate for the lack of '$G\alpha_z$ signalling complexes', resulting in a similar functional

outcome in those behavioural responses that exhibit a functional receptor reserve (such as acute morphine lethality or analgesia) (Sora et al., 2001a). However, as $G\alpha_{i/o}$ and $G\alpha_z$ differ biochemically (Casey et al., 1990), the resultant '$G\alpha_{i/o}$ and $G\alpha_z$ signalling complexes' may possess different properties (e.g. desensitisation or internalisation properties), such that the '$G\alpha_{i/o}$ signalling complex' is no longer able to compensate for the absence of the '$G\alpha_z$ signalling complex' after chronic morphine treatment.

7.2 Compensation at the system level

In addition to compensation by other molecules within the same cell, the chronic absence of a protein as a result of gene targeting may trigger compensatory changes in other parts of the system linked to the function of the protein. For example, in mice lacking the norepinephrine transporter, there was an enhancement of post-synaptic D2/D3 receptor function (Xu et al., 2000a). This was explained by the authors to be due to downregulation of presynaptic dopamine neurotransmission, which in turn may be indirectly attributed to the possible absence of dopamine clearance by the transporter in the region of the dopamine cell body (Xu et al., 2000a). Although this form of compensation can potentially happen in the studies of all gene knockout mice, due to its complexity, it has rarely been reported. However, given the low level of expression of $G\alpha_z$ relative to $G\alpha_{i/o}$ in the brain (Sternweis and Robishaw, 1984) and most of $G\alpha_z$'s signalling functions also appeared to be performed by $G\alpha_{i/o}$ (Ho and Wong, 1998), the effect of $G\alpha_z$ inactivation may generally not be large enough to trigger system wide compensation.

7.3 Assignment of the signalling pathway affected by the absence of $G\alpha_z$

G protein coupled receptors (GPCRs) comprise one of the largest and most diverse superfamily of proteins in our body, with over a thousand members (Bockaert et al., 2002). In contrast, there are only an estimated twenty seven G protein α subunits in the human genome (Venter et al., 2001). Hence, with the exception of a few highly specialized G protein α subunits with restricted tissue distribution (Table 1.1), most G protein α subunits are likely to couple to quite a number of different receptors.

Although $G\alpha_z$ appears to have a relatively limited tissue distribution (Chapter 1, Section 1.3), *in vitro* experiments have suggested that it can couple to more than fifteen different GPCRs (Chapter 1, Section 1.4.1), and has the potential of coupling to all $G\alpha_{i/o}$ coupled receptors when over-expressed (Ho and Wong, 1998). Accordingly, disruption of the gene coding for $G\alpha_z$, may cause the functions of a number of GPCRs to be impaired. However, most of this impairment of GPCR function is likely to be only partial since $G\alpha_i$ and $G\alpha_o$ are believed to normally play a role in transducing the activation signal of these GPCRs as well.

In this thesis, I have focussed on examining whether G_z is involved in transducing the signals caused by stimulation of opioid receptors, as well as activation of the dopamine D2-like family of receptors. Due to limitation in the number of age and gender matched $G\alpha_z$ knockout and wildtype control mice available for experimentation (caused primarily by problems with $G\alpha_z$ knockout mice breeding in the C57BL/6 background) (Kelleher, 2000), I have chosen morphine as the agonist for investigation of the opioid receptor due to its clinical relevance and its purported 'G_z preferring characteristics' (Garzon et al., 1998) in order to maximize the chances of seeing an effect. Although morphine can bind to μ, δ and κ receptors, it has the highest affinity for the μ receptor (Goldstein and Naidu, 1989; Raynor et al., 1994). In an attempt to define the particular opioid receptor that produce an effect in the $G\alpha_z$ knockout animals, I have tried to employ morphine within doses where receptor knockout studies have shown it to be receptor specific for the particular effect being investigated. For instance, it is possible that 100mg/kg of morphine may still act specifically at the μ receptor for the hypothermic response to morphine since this dose of morphine produced hypothermia in wildtype animals that was abolished in the μ receptor knockout mouse (Sarton et al., 2001). In instances where a difference between wildtype and $G\alpha_z$ knockout mice was observed only at a higher morphine dose, which can potentially be due to a large reserve of $G_{i/o}$ coupled receptors present, I attempted to rule out that the effect seen was not occurring at another opioid receptor by using an agonist specific for that receptor (e.g. the greater locomotor activation by morphine in the $G\alpha_z$ knockout mouse is not due to morphine acting at the κ receptor). Although this approach is not fool-proof, my ability to experiment with other opioid agonists is limited by the number of animals available to me and at times by the availability of the drug (which sometimes require importation and possession permits) or the injection technique required (e.g. intracerebroventricular

injection in mice). For the dopamine receptor experiments, quinpirole was chosen as its effects on dopamine D2-like receptors is very well characterized. Nonetheless, there are plans to extend the studies reported here to other dopamine D2-like agonists once the animals become available.

Given the problem that a deficiency of $G\alpha_z$ is likely to affect a number of different GPCRs, and behavioural outcomes are the result of activation of neuronal networks that typically involve multiple receptors, the use of multiple agonists acting at the same receptor to investigate one particular behavioural phenomenon would still not prove that the receptor is coupled to G_z. In an attempt to define the particular signalling pathway affected by the absence of G_z, the approach employed here was to investigate the system with a variety of different measures, each of which has been clearly established to be mediated by stimulation of a particular receptor in receptor knockout studies. For instance, the μ opioid receptor is known to mediate morphine analgesia, physical dependence (Matthes et al., 1996) and lethality (Loh et al., 1998), as well as the hypothermic (Sarton et al., 2001) and locomotor stimulating (Sora et al., 2001a) effects of the drug in mice. Accordingly, I have examined the responses of $G\alpha_z$ knockout mice to morphine on all of these measures. Assuming that G_z and $G_{i/o}$ are both coupled to the μ opioid receptor across the variety of neuronal networks that produce these distinct behavioural outcomes, I would expect the absence of $G\alpha_z$ to be felt in some way in all of these different measures. Indeed, I found that morphine analgesia and lethality are decreased in $G\alpha_z$ knockout mice after the development of morphine tolerance. The hypothermic response to morphine was also attenuated in mutant mice, and the duration of acute morphine analgesia and locomotor activation were also slightly reduced. Similarly, the coupling of D2-like receptors to G_z was demonstrated by an attenuation of quinpirole induced hypothermia, ACTH release and locomotor activity depression, all of which are known to be mediated through stimulation of dopamine D2-like receptors. To further strengthen the findings, I also showed that the quinpirole inhibition of dopamine release caused by stimulation of the medial forebrain bundle is significantly reduced in $G\alpha_z$ knockout mice.

An unexpected result that was produced from the use of such an approach is the increase in locomotor stimulation during the early phase of morphine stimulation. This demonstrates the caveat of using just one behavioural measure and the general employment of behavioural outcomes as a way of defining the receptor that G_z is

coupled to since an effect of the absence of $G\alpha_z$ could be due to an impairment downstream from where the agonist acts. Fortunately, in this case, the neuronal circuitry involved in morphine induced locomotor stimulation is quite well known, and it is possible that the greater locomotor stimulation caused by morphine in the $G\alpha_z$ knockout animals is due to an impairment of downstream dopamine D2-like receptors.

Given the difficulties with interpretation of behavioural outcomes and the limitation in the number of $G\alpha_z$ knockout mice available, a more direct biochemical or electrophysiological demonstration of G_z coupling to these receptors would have been preferred. The biochemical approach has been pursued relentlessly by my predecessor (Dr. Kim Powell nee Kelleher) and myself for more than a year. However, we were not able to observe any reliable difference between wildtype and $G\alpha_z$ knockout mice in membrane preparations. Thus far, the only biochemical demonstration of G_z producing an effect *in vivo*, is its ability to mediate epinephrine induced inhibition of cyclic AMP formation (Yang et al., 2000) and activation of Rap1 (Woulfe et al., 2002) in intact blood platelets, where $G\alpha_z$ is the second most abundant G protein α subunit after $G\alpha_{i2}$ (Woulfe et al., 2002). One of the major problems with working on G_z in brain membrane preparations is the relative abundance of $G_{i/o}$, which is expected to easily compensate for the absence of G_z once membrane compartments are destroyed (Allgeier et al., 1997; Degtiar et al., 1997).

On the other hand, the pursuit of an electrophysiological approach to the study of G_z function has been hampered by the absence of good hypotheses of the role performed by G_z and data on its precise localization in the brain (e.g. nerve terminal versus cell body). In this respect, the data presented in the present thesis may serve as a useful starting point for the further examination of G_z's functions in the brain via electrophysiology. $G\alpha_z$ has been reported to be widely present in many parts of the brain (albeit at a low concentration relative to $G\alpha_{i/o}$) (see section 1.3). Currently, we are in the process of developing an antibody to verify these findings using the $G\alpha_z$ knockout mouse, and to more precisely locate the particular populations of neurons that contain $G\alpha_z$.

7.4 Future directions

All the data presented in this thesis were based on the study of wildtype and $G\alpha_z$ knockout mice in pure C57BL/6 genetic background. The use of a pure genetic background avoids the 'hitchhike donor gene' confound that is often associated with comparing knockout and wildtype littermates in a mixed genetic background, where phenotypic differences may be attributed to a background gene that 'hitchhikes' along with the mutated gene by virtue of their positional proximity on the chromosome (Lariviere et al., 2001). Despite this major advantage, the generalizability of these results to mice of other strains and ultimately to humans will need to be tested. For certain physiological processes like thermoregulation, widespread strain and species differences are known to exist (Adler and Geller, 1993; Belknap et al., 1998; Lipton and Clark, 1986). To address the issue of strain variation, my laboratory has just successfully created a congenic line of $G\alpha_z$ knockout mice in the BALB/c background through twenty repeated back-crossings with wildtype BALB/c mice. These N20 BALB/c $G\alpha_z$ knockout mice will be useful in the future for assessing the generalizability of the current results.

Despite some of the limitations of a gene knockout approach as discussed in the earlier sections, the technique allows a very 'clean' and precise inactivation of the $G\alpha_z$ protein, which would not be achievable by the use of other methods (antibodies, antisense oligonucleotides). This is probably one of the few methods currently available for studying an intracellular factor like $G\alpha_z$ *in vivo*, which shares about 66-67% amino acid identity with its more abundant $G\alpha_i$ relatives (Simon et al., 1991). To confirm that the phenotypes observed in $G\alpha_z$ knockout mice were due to the absence of $G\alpha_z$ and not a result of developmental abnormality in mutant mice, I have also attempted to reconstitute the $G\alpha_z$ knockout mouse by making transgenic mice that over-express $G\alpha_z$. I have made two constructs, one containing a mutationally activated form of $G\alpha_z$ ($G\alpha_z$ Q205L) (Wong et al., 1992), and one containing wildtype $G\alpha_z$, both under the control of the human elongation factor 1α promoter (Kim et al., 1990; Uetsuki et al., 1989). Both of these constructs also contain an internal ribosomal entry site (Wong et al., 2002), which allows the co-expression of $G\alpha_z$ with Enhanced Green Fluorescent Protein (EGFP) (Zhang et al., 1996; Zhu et al., 1999a) under the same promoter. The constructs were linearised and electroporated into mouse embryonic stem (ES) cells. A number of

'green' clones were selected and verified to contain the targeting construct by polymerase chain reaction (Mullis et al., 1986). They were further shown to express $G\alpha_z$ / $G\alpha_z$ Q205L and EGFP by immunohistochemistry and western blot analysis. When these 'verified' clones were injected into blastocysts and implanted into pseudopregnant female mice, less than ten 'green' chimaera were born despite a total of twenty injections over a one year period (It is of note that the same ES cells transfected with other knockout or transgenic constructs routinely produced germline chimaera over the same time period, indicating that the ES cell line used was fully competent). Of the 'green' chimaera that were produced, most perished within 3 weeks of age and appeared larger in size compared to 'non-green' littermates. The few that survived for longer than three weeks were infertile.

Since the over-expression of $G\alpha_z$ in all tissues under the human elongation factor 1α promoter appears to result in early developmental lethality, I attempted to circumvent this problem by collaborating with Dr Kyoko Koishi (University of Otago, New Zealand) to generate a 'conditional' transgenic mouse, which would express $G\alpha_z$ only in neurons after the animals have been fed with the tetracycline analog, doxycycline (Kistner et al., 1996). This mouse is currently still being produced. When this mouse is finally made, my laboratory intends to cross the $G\alpha_z$ over-expressing transgenic mouse with the $G\alpha_z$ knockout mouse. This would result in a $G\alpha_z$ knockout mouse that would make $G\alpha_z$ only after it has been fed with doxycycline. The mouse would be very useful for certain behavioural experiments as it would allow the same mouse to serve as its own control.

7.5 General Conclusion

In Chapter 3, I have shown that G_z plays an important role in mediating the supraspinal analgesic and lethality effects of morphine after tolerance development. The greater morphine tolerance that developed in the $G\alpha_z$ knockout animals was not due to pharmacokinetic or behavioural mechanisms. I have also demonstrated a gene dose dependent reduction in naloxone precipitated jumping in the $G\alpha_z$ knockout mouse. My data provides strong support for a dissociation between the different signs of physical dependence, indicating that morphine withdrawal is not a unitary phenomenon. It is therefore very important when measuring the degree of physical dependence, to consider a range of different withdrawal signs. In addition, the dissociation between

morphine tolerance and naloxone precipitated jumping suggests that different G_z signalling pathways are involved in mediating the effects of the loss of $G\alpha_z$ on morphine tolerance and physical dependence. Clinically, it may be possible to selectively affect these separate pathways to prevent the development of morphine tolerance and physical dependence in human patients.

In Chapter 4, I have shown that G_z is involved in the regulation of morphine stimulated locomotor responses. The locomotor activating effects of psychostimulants are often related to their rewarding effects (Wise, 1987). G_z may therefore play a role in determining the vulnerability of drug users to become dependent on addictive drugs.

In Chapter 5, I have provided evidence that G_z can couple to D2-like receptors *in vivo*. Since disturbances in dopaminergic networks are implicated in a number of debilitating human conditions and diseases, including Parkinson's disease, schizophrenia, and drug addiction, G_z might serve as a potential drug target when subtle fine tuning of dopaminergic functions are required.

In Chapter 6, I have shown that morphine induced hypothermia, a major undesirable side effect of morphine during its surgical use, is significantly attenuated in mice lacking $G\alpha_z$, and therefore suggests a potential site for therapeutic clinical intervention. However, the mechanism underlying this phenomenon is currently restricted to this mouse strain and whether the inactivation of $G\alpha_z$ mediated signal transduction pathways can prevent morphine hypothermia in other mouse strains and humans would need to be further explored.

Finally, this thesis has furnished valuable insights about the organization of cellular signalling in intact cells. These insights provide a succinct explanation for many of the discrepant findings on G protein coupling in the litterature, depending whether the studies were performed using broken membrane preparations or in transfected cell lines. By providing a theoretical framework, these insights lay the foundation for future discoveries about the functions of G_z and other signalling molecules *in vivo*.

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